PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

B2

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/32, A01N 63/02

A2

(11) International Publication Number: WO 91/14778

(43) International Publication Date: 3 October 1991 (03.10.91)

(21) International Application Number: PCT/US91/01786

(22) International Filing Date: 18 March 1991 (18.03.91)

(30) Priority data:
496,568 20 March 1990 (20.03.90) US

(71) Applicant: ECOGEN INC. [US/US]; 2005 Cabot Boulevard West, Langhorne, PA 19047-1810 (US).

(72) Inventors: DONOVAN, William, P. ; 36 Calicobush Road, Levittown, PA 19057 (US). RUPAR, Mark, J. ; 2225-C Prior Road, Wilmington, DE 19809 (US). SLANEY, Annette, C. ; 4 Dunmoor Court South, Hamilton Square, NJ 08690 (US). JOHNSON, Timothy, B. ; 142 E. Highland Avenue, Langhorne, PA 19047 (US).

(74) Agents: NADEL, Alan, S.; Panitch, Schwarze, Jacobs & Nadel, 1601 Market Street, 36th Floor, Philadelphia, PA 19103 (US) et al.

(81) Designated States: AT (European patent), AU, BE (European patent), BR, CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent), SU.

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: *BACILLUS THURINGIENSIS cryIIIC* GENE AND PROTEIN TOXIC TO COLEOPTERAN INSECTS

(57) Abstract

A purified and isolated *cryIII*-type gene was obtained from a novel *B.t.* strain. The gene has a nucleotide base sequence coding for the amino acid sequence illustrated in Figure 1. The 74.4 kDa protein produced by this gene is an irregularly shaped crystal that is toxic to coleopteran insects, including Colorado potato beetle and insects of the genus *Diabrotica*.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the *PCT*.

AT	Austria	**ES**	Spain	**MG**	Madagascar
AU	Australia	**FI**	Finland	**ML**	Mali
BB	Barbados	**FR**	France	**MN**	Mongolia
BE	Belgium	**GA**	Gabon	**MR**	Mauritania
BF	Burkina Faso	**GB**	United Kingdom	**MW**	Malawi
BG	Bulgaria	**GN**	Guinea	**NL**	Netherlands
BJ	Benin	**GR**	Greece	**NO**	Norway
BR	Brazil	**HU**	Hungary	**PL**	Poland
CA	Canada	**IT**	Italy	**RO**	Romania
CF	Central African Republic	**JP**	Japan	**SD**	Sudan
CG	Congo	**KP**	Democratic People's Republic of Korea	**SE**	Sweden
CH	Switzerland			**SN**	Senegal
CI	Côte d'Ivoire	**KR**	Republic of Korea	**SU**	Soviet Union
CM	Cameroon	**LI**	Liechtenstein	**TD**	Chad
CS	Czechoslovakia	**LK**	Sri Lanka	**TG**	Togo
DE	Germany	**LU**	Luxembourg	**US**	United States of America
DK	Denmark	**MC**	Monaco		

BACILLUS THURINGIENSIS cryIIIC GENE AND PROTEIN TOXIC TO COLEOPTERAN INSECTS

Field of the Invention

The present invention relates to a gene isolated from Bacillus thuringiensis (hereinafter "B.t.") encoding an insecticidal crystal protein designated CryIIIC, as well as insecticidal compositions containing the protein and plants transformed with the gene. The insecticidal compositions and transformed plants are toxic to insects of the order Coleoptera, and are particularly toxic to insects of the genus Diabrotica.

Background of the Invention

B.t. is a gram-positive soil bacterium that produces crystal proteins during sporulation which are specifically toxic to certain orders and species of insects. Many different strains of B.t. have been shown to produce insecticidal crystal proteins. Compositions including B.t. strains which produce insecticidal proteins have been commercially available and used as environmentally acceptable insecticides because they are quite toxic to the specific target insect, but are harmless to plants and other non-targeted organisms.

A number of genes encoding crystal proteins have been cloned from several strains of B.t. A good overview is set forth in H. Hofte et al., Microbiol. Rev., 53, pp. 242-255 (1989). While this reference is not prior art with respect to the present invention, it provides a good overview of the genes and proteins obtained from B.t. and their uses, adopts a nomenclature and classification scheme for B.t. genes and proteins, and has an extensive bibliography.

The B.t. crystal protein is active in the insect only after ingestion. After ingestion by an insect, the alkaline pH and proteolytic enzymes in the mid-gut solubilize the crystal allowing the release of the toxic components. These toxic components disrupt the mid-gut cells causing the insect to cease feeding and, eventually, to die. In fact, B.t. has proven to be an effective and environmentally safe insecticide in dealing with various insect pests.

As noted by Hofte et al., the majority of insecticidal B.t. strains are active against insects of the order Lepidoptera, i.e., caterpillar insects. Other B.t. strains are insecticidally active against insects of the order Diptera, i.e., flies and mosquitoes, or against both lepidopteran and dipteran insects. In recent years, a few B.t. strains have been reported as producing crystal protein that is insecticidal to insects of the order Coleoptera, i.e., beetles.

The first isolation of a coleopteran-toxic B.t. strain is reported by A. Krieg et al., in Z. angew. Ent., 96, pp. 500-508 (1983); see

also A. Krieg et al., Anz. Schaedlingskde, Pflanzenschutz, Umweltschutz, 57, pp. 145-150 (1984) and U.S. Patent 4,766,203, issued August 23, 1988 of A. Krieg et al. The strain, designated B.t. var. tenebrionis, is reported to be toxic to larvae of the coleopteran insects Agelastica alni (blue alder leaf beetle) and Leptinotarsa decemlineata (Colorado potato beetle). B.t. tenebrionis makes an insecticidal crystal protein reported to be about 65-70 kilodaltons (kDa) (U.S. Patent 4,766,203; see also K. Bernhard, FEMS Microbiol. Lett. 33, pp. 261-265 (1986).

V. Sekar et al., Proc. Natl. Acad. Sci. USA, 84, pp. 7036-7040 (1987), report the cloning and characterization of the gene for the coleopteran-toxic crystal protein of B.t. tenebrionis. The size of the protein, as deduced from the sequence of the gene, was 73 kDa, but the isolated protein contained primarily a 65 kDa component. Hofte et al., Nucleic Acids Research, 15, p. 7183 (1987), also report the DNA sequence for the cloned gene from B.t. tenebrionis, and the sequence of the gene is identical to that reported by Sekar et al. (1987).

McPherson et al., Bio/Technology, 6, pp. 61-66 (1988), disclose the DNA sequence for the cloned insect control gene from B.t. tenebrionis, and the sequence is identical to that reported by Sekar et al. (1987). E. coli cells and Pseudomonas fluorescens cells harboring the cloned gene were found to be toxic to Colorado potato beetle larvae.

A coleopteran-toxic strain, designated B.t. var. san diego, is reported by C. Herrnstadt et al., Bio/Technology, 4, pp. 305-308 (1986), to produce a 64 kDa crystal protein that was toxic to various coleopteran insects: strong toxicity to Pyrrhalta luteola (elm leaf beetle); moderate toxicity to Anthonomus grandis (boll weevil), Leptinotarsa decemlineata (Colorado potato beetle), Otiorhynchus sulcatus (black vine weevil), Tenebrio molitor (yellow mealworm) and Haltica tombacina; and weak toxicity to Diabrotica undecimpunctata undecimpunctata (western spotted cucumber beetle).

The DNA sequence of the cloned coleopteran toxin gene of B.t. san diego is reported in C. Herrnstadt et al., Gene, 57, pp. 37-46 (1987); see also U.S. Patent 4,771,131, issued September 13, 1988, of Herrnstadt et al. The sequence of the toxin gene of B.t. san diego is identical to that reported by Sekar et al. (1987) for the cloned coleopteran toxin gene of B.t. tenebrionis.

A. Krieg et al., J. Appl. Ent., 104, pp. 417-424 (1987), report that the strain B.t. san diego is identical to the B.t. tenebrionis strain, based on various diagnostic tests.

Another new B.t. strain, designated EG2158, is reported by W. P. Donovan et al., Mol. Gen. Genet., 214 pp. 365-372 (1988) to produce a 73 kDa crystal protein that is insecticidal to coleopteran insects. The toxin-encoding gene from B.t. strain EG2158 was cloned and sequenced, and its sequence is identical to that reported by Sekar et al. (1987) for the cloned B.t. tenebrionis

coleopteran toxin gene. This coleopteran toxin gene is referred to as the cryIIIA gene by Höfte et al., Microbiol. Rev., 53, pp. 242-255 (1989).

U.S. Patent 4,797,279, issued January 10, 1989, of D. Karamata et al., discloses a hybrid B.t. microorganism containing a plasmid from B.t. kurstaki with a lepidopteran toxin gene and a plasmid from B.t. tenebrionis with a coleopteran toxin gene. The hybrid B.t. produces crystal proteins characteristic of those made by B.t. kurstaki, as well as of B.t. tenebrionis.

European Patent Application Publication No. 0 303 379, published February 15, 1989, of Mycogen Corporation, discloses a novel B.t. isolate identified as B.t. MT 104 which has insecticidal activity against both coleopteran and lepidopteran insects.

European Patent Application Publication No. 0 318 143, published May 31, 1989, of Lubrizol Genetics, Inc., discloses the cloning, characterization and selective expression of the intact partially modified gene from B.t. tenebrionis, and the transfer of the cloned gene into a host microorganism rendering the microorganism able to produce a protein having toxicity to coleopteran insects. Insect bioassay data for B.t. san diego reproduced from Herrnstadt et al., Bio/Technology, 4, pp. 305-308 (1986) discussed above, is summarized. The summary also includes data for B.t. tenebrionis, from another source; B.t. tenebrionis is reported to exhibit strong toxicity to Colorado potato beetle, moderate

toxicity to western corn rootworm (Diabrotica virgifera virgifera) and weak toxicity to southern corn rootworm (Diabrotica undecimpunctata).

European Patent Application Publication No. 0 324 254, published July 19, 1989, of Imperial Chemical Industries PLC, discloses a novel B.t. strain identified as A30 which has insecticidal activity against coleopteran insects.

European Patent Application Publication No. 0 328 383, published August 16, 1989, of Mycogen Corporation, discloses a novel B.t. microorganism identified as B.t. PS40D1 which has insecticidal activity against coleopteran insects.

European Patent Application Publication No. 0 330 342, published August 30, 1989, of Mycogen Corporation, discloses a novel B.t. microorganism identified as B.t. PS86B1 which has insecticidal activity against coleopteran insects.

These latter four publications are not prior art with respect to the present invention.

B.t. tenebrionis, first reported by A. Krieg et al., was discovered in or near Darmstadt, Germany and it is believed that B.t. san diego, reported by Herrnstadt et al., was obtained from a location in or near San Diego, California. B.t. strain EG2158, reported by Donovan et al., was isolated from a sample of crop dust from Kansas. Thus, various B.t. strains that have been isolated from several widely separated geographical locations all contained an apparently identical coleopteran toxin gene, the cryIIIA gene.

There appear to be no reports in the literature of any new coleopteran toxin B.t. genes other than the unique B.t. gene first discovered in B.t. tenebrionis over seven years ago.

Moreover, even among the various B.t. strains that have been reported as having crystal proteins insecticidally active against coleopteran insects, none has been shown to have significant toxicity to the larvae and adults of the insect genus Diabrotica (corn rootworm), which includes the western corn rootworm (Diabrotica virgifera virgifera), the southern corn rootworm (Diabrotica undecimpunctata howardi) and the northern corn rootworm (Diabrotica barberi). The cryIIIC gene of the present invention expresses protein toxin having quantifiable insecticidal activity against the Diabrotica insects, among other coleopteran insects.

Summary of the Invention

One aspect of the present invention relates to a purified and isolated coleopteran toxin gene having a nucleotide base sequence coding for the amino acid sequence illustrated in Figure 1 and hereinafter designated as the cryIIIC gene. The cryIIIC gene has a coding region extending from nucleotide bases 14 to 1972 shown in Figure 1.

Another aspect of the present invention relates to the insecticidal protein produced by the cryIIIC gene. The CryIIIC protein has the amino acid sequence, as deduced from the nucleotide sequence of the cryIIIC gene from bases 14 to 1972, that is shown in Figure 1. The protein exhibits

insecticidal activity against insects of the order Coleoptera, in particular, Colorado potato beetle and insects of the genus Diabrotica.

Still another aspect of the present invention relates to a biologically pure culture of a B.t. bacterium deposited with the NRRL having Accession No. NRRL B-18533 and being designated as B.t. strain EG4961. B.t. strain EG4961 carries the cryIIIC gene and produces the insecticidal CryIIIC protein. Biologically pure cultures of other B.t. bacteria carrying the cryIIIC gene are also within the scope of this invention.

Yet another aspect of this invention relates to insecticidal compositions containing, in combination with an agriculturally acceptable carrier, either the CryIIIC protein or fermentation cultures of a B.t. strain which has produced the CryIIIC protein.

The invention also includes a method of controlling coleopteran insects by applying to a host plant for such insects an insecticidally effective amount of the CryIIIC protein or of a fermentation culture of a B.t. strain that has made the CryIIIC protein. The method is applicable to a variety of coleopteran insects, including Colorado potato beetle, elm leaf beetle, imported willow leaf beetle and corn rootworm.

Still another aspect of the present invention relates to a recombinant plasmid containing the cryIIIC gene, a biologically pure culture of a bacterium transformed with such

recombinant plasmid, the bacterium preferably being B.t., as well as a plant transformed with the cryIIIC gene.

A further aspect of the present invention relates to a method of enhancing the insecticidal activity against coleopteran insects of an insecticidal composition containing a coleopteran-toxic protein, where the method comprises adding to, or incorporating into, the composition containing a CryIII protein a CryI protein in an amount effective to enhance the insecticidal activity of the composition. Insecticidal compositions containing the CryIIIC protein and a CryI protein exhibit enhanced insecticidal activity is against insects of the order Coleoptera, particularly Colorado potato beetle and corn rootworm.

Brief Description of the Drawings

Figure 1 comprises Figures 1-1 through 1-3 and shows the nucleotide base sequence of the cryIIIC gene and the deduced amino acid sequence of the CryIIIC protein. The putative ribosome binding site (RBS) is indicated. HindIII and BamHI restriction sites are also indicated.

Figure 2 is a photograph of an ethidium bromide stained agarose gel containing size fractionated native plasmids of B.t. strains EG2158, EG2838 and EG4961. The numbers to the right of Figure 2 indicate the approximate sizes, in megadaltons (MDa), of the plasmids of B.t. strain EG4961.

Figure 3 is a photograph of an autoradiogram made by transferring the plasmids shown in Figure 2 to a nitrocellulose filter, hybridizing the filter with a radioactively labeled 2.4 kilobase (kb) cryIIIB probe, and exposing the filter to X-ray film. The number to the right of Figure 3 indicates the size, in MDa, of the plasmid of B.t. strain EG4961 that hybridizes to the cryIIIB probe. The letter "f" to the right of Figure 3 indicates the fragments that result from the breakdown of the cryIIIB-hybridizing plasmid.

Figure 4 is a photograph of an ethidium bromide stained agarose gel containing DNA from B.t. strains EG2158, EG2838 and EG4961 that has been digested with HindIII plus EcoRI and size fractionated by electrophoresis. The lane labeled "stnd" is a size standard.

Figure 5 is a photograph of an autoradiogram made by transferring the DNA fragments of Figure 4 to a nitrocellulose filter, hybridizing the filter with the radioactively labeled 2.4 kb cryIIIB probe, and exposing the filter to X-ray film. The numbers to the right of Figure 5 indicate the sizes, in kb, of B.t. strain EG4961 restriction fragments that hybridize to the cryIIIB probe. The lane labeled "stnd" is a size standard.

Figure 6 is a photograph of a Coomassie stained sodium dodecyl sulfate ("SDS") polyacrylamide gel showing crystal proteins solubilized from B.t. strains EG2158, EG2838 and

EG4961. The numbers to the right of Figure 6 indicate the approximate sizes in kDa of the crystal proteins produced by B.t. strain EG4961.

Figure 7 shows a restriction map of plasmid pEG258. The location and orientation of the cryIIIC gene is indicated by an arrow. A gene designated the cryX gene is located within the region indicated by the dotted line. Asp stands for Asp718, Bam stands for BamHI, H3 stands for HindIII and P stands for PstI restriction enzymes. A one kb scale marker is also illustrated.

Figure 8, aligned with and based on the same scale as Figure 7, shows a restriction map of plasmid pEG260 containing an 8.3 kb fragment of DNA from B.t. strain EG4961 where the cryIIIC gene is indicated by an arrow and the cryX gene is located within the region indicated by the dotted line. In addition to the abbreviations for the restriction enzymes set forth above regarding Figure 7, (RV/Asp) stands for the fusion of EcoRV and Asp718 restrictions sites, and (RV/Pst) stands for the fusion of EcoRV and PstI restriction sites.

Figure 9, aligned with and based on the same scale as Figure 7, shows a restriction map of plasmid pEG269 containing the cryIIIC gene as indicated by an arrow, as part of a fragment of DNA from recombinant E. coli strain EG7233. The abbreviations used with regard to pEG258 illustrated in Figure 7 are applicable to this figure. In addition, Sph stands for the SphI restriction site, and S3A/Bam stands for the fusion of Sau3IIIA and BamHI restriction sites.

Figure 10 is a photograph of a Coomassie stained SDS-polyacrylamide gel. The gel shows protein bands synthesized by the following bacterial strains: E. coli strain EG7221(pUC18/Cry$^-$); E. coli strain EG7218(pEG258/cryIIIC$^+$ cryX$^+$); B.t. strain EG7211(pEG220/Cry$^-$); B.t. strain EG4961(cryIIIC$^+$ cryX$^+$); B.t. strain EG7231(pEG269/cryIIIC+ cryX$^-$); and B.t. strain EG7220(pEG260/cryIIIC$^+$ cryX$^+$). The numbers to the right of the gel indicate approximate sizes, in kDa, of the crystal proteins produced by these strains.

Detailed Description of the Preferred Embodiments

The isolation and purification of the cryIIIC gene and the coleopteran-toxic CryIIIC crystal protein and the characterization of the new B.t. strain EG4961 which produces the CryIIIC protein are described at length in the Examples. The utility of B.t. strain EG4961 and of the CryIIIC crystal protein in insecticidal compositions and methods is also illustrated in the Examples.

The Examples also illustrate the synergistic enhancement of the insecticidal activity of CryIII protein by the addition of a CryI protein. Thus, insecticidal compositions having a combination of both CryIII and CryI proteins provide enhanced insecticidal activity, particularly with respect to both larvae and adult Colorado potato beetle and southern corn rootworm, as well as other insects.

The cryIII-type gene of this invention, the cryIIIC gene, has the nucleotide base sequence shown in Figure 1. The coding region of the cryIIIC gene extends from nucleotide base position 14 to position 1972 shown in Figure 1.

A comparison of the nucleotide base pairs of the cryIIIC gene coding region with the corresponding coding region of the prior art cryIIIA gene indicates significant differences between the two genes. The cryIIIC gene is only 75% homologous (positionally identical) with the cryIIIA gene.

A comparison of the nucleotide base pairs of the cryIIIC gene coding region with the corresponding coding region of the cryIIIB gene obtained from recently discovered B.t. strain EG2838 (NRRL Accession No. B-18603) indicates that the cryIIIC gene is 96% homologous (positionally identical) with the cryIIIB gene.

The CryIII-type protein of this invention, the CryIIIC protein, that is encoded by the cryIIIC gene, has the amino acid sequence shown in Figure 1. In this disclosure, references to the CryIIIC "protein" are synonymous with its description as a "crystal protein", "protein toxin", "insecticidal protein" or the like, unless the context indicates otherwise. The size of the CryIIIC protein, as deduced from the DNA sequence of the cryIIIC gene, is 74.4 kDa.

The size of the CryIIIB protein, as deduced from the sequence of the cryIIIB gene, is 74.2 kDa. The prior art CryIIIA protein, encoded by the cryIIIA gene, has a deduced size of 73.1 kDa.

Despite the apparent size similarity, comparison of the amino acid sequence of the CryIIIC protein with that of the prior art CryIIIA protein shows significant differences between the two. The CryIIIC protein is only 69% homologous (positionally identical amino acids) with the CryIIIA protein. The CryIIIC protein is 94% homologous with the CryIIIB protein. Nevertheless, despite the apparent homology of the CryIIIC and CryIIIB proteins, the CryIIIC protein has been shown to be a different protein than the CryIIIB protein, based on its significantly improved insecticidal activity compared to the CryIIIB protein with respect to insects of the order Coleoptera and in particular, insects of the genus Diabrotica. The CryIIIC protein is the first B.t. protein to exhibit quantifiable insecticidal activity against corn rootworms.

The present invention is intended to cover mutants and recombinant or genetically engineered derivatives of the cryIIIC gene that yield a coleopteran-toxic protein with essentially the same properties as the CryIIIC protein.

The cryIIIC gene is also useful as a DNA hybridization probe, for discovering similar or closely related cryIII-type genes in other B.t. strains. The cryIIIC gene, or portions or derivatives thereof, can be labeled for use as a

hybridization probe, e.g., with a radioactive label, using conventional procedures. The labeled DNA hybridization probe may then be used in the manner described in the Examples.

The cryIIIC gene and the corresponding insecticidal CryIIIC protein were first identified in B.t. strain EG4961, a novel B.t. strain. The characteristics of B.t. strain EG4961 are more fully described in the Examples. Comparison of the plasmid arrays and other strain characteristics of B.t. strain EG4961 with those of the recently discovered B.t. strain EG2838 and those of the prior art B.t. strain EG2158 demonstrates that these three coleopteran-toxic B.t. strains are distinctly different.

The cryIIIC gene may be introduced into a variety of microorganism hosts, using procedures well known to those skilled in the art for transforming suitable hosts under conditions which allow for stable maintenance and expression of the cloned cryIIIC gene. Suitable hosts that allow the cryIIIC gene to be expressed and the CryIIIC protein to be produced include Bacillus thuringiensis and other Bacillus species such as B. subtilis or B. megaterium. It should be evident that genetically altered or engineered microorganisms containing the cryIIIC gene can also contain other toxin genes present in the same microorganism and that these genes could concurrently produce insecticidal crystal proteins different from the CryIIIC protein.

The Bacillus strains described in this disclosure may be cultured using conventional growth media and standard fermentation techniques. The B.t. strains harboring the cryIIIC gene may be fermented, as described in the Examples, until the cultured B.t. cells reach the stage of their growth cycle when CryIIIC crystal protein is formed. For sporogenous B.t. strains, fermentation is typically continued through the sporulation stage when the CryIIIC crystal protein is formed along with spores. The B.t. fermentation culture is then typically harvested by centrifugation, filtration or the like to separate fermentation culture solids, containing the CryIIIC crystal protein, from the aqueous broth portion of the culture.

The B.t. strains exemplified in this disclosure are sporulating varieties (spore forming or sporogenous strains) but the cryIIIC gene also has utility in asporogenous Bacillus strains, i.e., strains that produce the crystal protein without production of spores. It should be understood that references to "fermentation cultures" of B.t. strains (containing the cryIIIC gene) in this disclosure are intended to cover sporulated B.t. cultures, i.e., B.t. cultures containing the CryIIIC crystal protein and spores, and sporogenous Bacillus strains that have produced crystal protein during the vegetative stage, as well as asporogenous Bacillus strains containing the cryIIIC gene in which the culture has reached the growth stage where crystal protein is actually produced.

The separated fermentation solids are primarily CryIIIC crystal protein and B.t. spores, along with some cell debris, some intact cells, and residual fermentation medium solids. If desired, the crystal protein may be separated from the other recovered solids via conventional methods, e.g., sucrose density gradient fractionation. Highly purified CryIIIC protein may be obtained by solubilizing the recovered crystal protein and then reprecipitating the protein from solution.

The CryIIIC protein, as noted earlier, is a potent insecticidal compound against coleopteran insects, such as the Colorado potato beetle, elm leaf beetle, imported willow leaf beetle, and the like. The CryIIIC protein, in contrast to the CryIIIA and CryIIIB proteins, exhibits measurable insecticidal activity against Diabrotica insects, e.g., corn rootworms, which have been relatively unaffected by other coleopteran-toxic B.t. crystal proteins. The CryIIIC protein may be utilized as the active ingredient in insecticidal formulations useful for the control of coleopteran insects such as those mentioned above. Such insecticidal formulations or compositions typically contain agriculturally acceptable carriers or adjuvants in addition to the active ingredient.

The CryIIIC protein may be employed in insecticidal formulations in isolated or purified form, e.g., as the crystal protein itself. Alternatively, the CryIIIC protein may be present in the recovered fermentation solids, obtained from culturing of a Bacillus strain, e.g., Bacillus thuringiensis, or other microorganism host carrying

the cryIIIC gene and capable of producing the CryIIIC protein. Preferred Bacillus hosts include B.t. strain EG4961 and genetically improved B.t. strains derived from B.t. strain EG4961. The latter B.t. strains may be obtained via plasmid curing and/or conjugation techniques and contain the native cryIIIC gene-containing plasmid from B.t. strain EG4961. Genetically engineered or transformed B.t. strains or other host microorganisms containing a recombinant plasmid that expresses the cloned cryIIIC gene, obtained by recombinant DNA procedures, may also be used.

Examples of such transformants include B.t. strains EG7231 and EG7220, both of which contain the cloned cryIIIC gene on a recombinant plasmid.

The recovered fermentation solids contain primarily the crystal protein and (if a sporulating B.t. host is employed) spores; cell debris and residual fermentation medium solids may also be present. The recovered fermentation solids containing the CryIIIC protein may be dried, if desired, prior to incorporation in the insecticidal formulation.

The formulations or compositions of this invention containing the insecticidal CryIIIC protein as the active component are applied at an insecticidally effective amount which will vary depending on such factors as, for example, the specific coleopteran insects to be controlled, the specific plant or crop to be treated and the method of applying the insecticidally active compositions.

An insecticidally effective amount of the insecticide formulation is employed in the insect control method of this invention.

The insecticide compositions are made by formulating the insecticidally active component with the desired agriculturally acceptable carrier. The formulated compositions may be in the form of a dust or granular material, or a suspension in oil (vegetable or mineral) or water or oil/water emulsions, or as a wettable powder, or in combination with any other carrier material suitable for agricultural application. Suitable agricultural carriers can be solid or liquid and are well known in the art. The term "agriculturally acceptable carrier" covers all adjuvants, e.g., inert components, dispersants, surfactants, tackifiers, binders, etc. that are ordinarily used in insecticide formulation technology; these are well known to those skilled in insecticide formulation.

The formulations containing the CryIIIC protein and one or more solid or liquid adjuvants are prepared in known manners, e.g., by homogeneously mixing, blending and/or grinding the insecticidally active CryIIIC protein component with suitable adjuvants using conventional formulation techniques.

The CryIIIC protein, and other coleopteran toxin proteins such as CryIIIB and CryIIIA, may also be used in combination with a CryI protein, to provide unexpectedly enhanced insecticidal activity against a coleopteran insect target. The coleopteran-specific activity of

CryIIIC, CryIIIB and CryIIIA proteins is greatly enhanced by the addition or incorporation of a CryI protein into an insecticidal composition containing such CryIII protein. This method may be employed to make synergistic CryIII-CryI protein insecticide compositions, via physical combination of the respective CryIII and CryI proteins or via combination of B.t. strains making the respective proteins. The preferred CryI protein for use in the synergistic CryIII insecticide combinations is CryIA, and particularly, CryIA(c), although it is believed that other CryI proteins can also be used in the synergistic combinations. Surprisingly, there appears to be no enhancement of the CryI protein's insecticidal efficacy against lepidopteran insects; i.e., there seems to be no "reverse synergy" with CryI proteins imparted by the presence of CryIII crystal proteins.

If desired, combinations of CryIIIC (or CryIIIB) and CryI proteins in this invention may be obtained in situ in combined form, from cultures of strains of B.t. or other microorganism hosts carrying such cryIII genes and cryI genes capable of producing the respective CryIII and CryI proteins. Such strains or hosts may be obtained via plasmid curing and/or conjugation techniques involving B.t. or other strains or host microorganisms containing a recombinant plasmid that expresses the cloned cryIII and cryI genes.

An amount of CryI protein approximately equivalent to the quantity of CryIII protein present in the composition provides good enhancement of coleopteran-specific insecticidal

activity. Smaller amounts of CryI protein than this 1:1 CryI:CryIII ratio will likely still give satisfactory levels of enhancement to the CryIII protein.

The insecticidal compositions of this invention are applied to the environment of the target coleopteran insect, typically onto the foliage of the plant or crop to be protected by conventional methods, preferably by spraying. Other application techniques, e.g., dusting, sprinkling, soaking, soil injection, seed coating, seedling coating or spraying, or the like, are also feasible and may be required for insects that cause root or stalk infestation. These application procedures are well known in the art.

The cryIIIC gene or its functional equivalent, hereinafter sometimes referred to as the "toxin gene," can be introduced into a wide variety of microorganism hosts. Expression of the cryIIIC gene results in the production of insecticidal CryIIIC crystal protein toxin. Suitable hosts include B.t. and other species of Bacillus, such as B. subtilis or B. megaterium, for example. Plant-colonizing or root-colonizing microorganisms may also be employed as the host for the cryIIIC gene. Various procedures well known to those skilled in the art are available for introducing the cryIIIC gene into the microorganism host under conditions which allow for stable maintenance and expression of the gene in the resulting transformants.

The transformants, i.e., host microorganisms that harbor a cloned gene in a recombinant plasmid, can be isolated in accordance with conventional methods, usually employing a selection technique, which allows growth of only those host microorganisms that contain a recombinant plasmid. The transformants then can be tested for insecticidal activity. Again, these techniques are standard procedures.

Characteristics of particular interest in selecting a host cell for purposes of production include ease of introducing the gene into the host, availability of expression systems, efficiency of expression, stability of the CryIIIC insecticidal protein in the host, and the presence of auxiliary genetic capabilities. The cellular host containing the insecticidal cryIIIC gene may be grown in any convenient nutrient medium, where expression of the cryIIIC gene is obtained and CryIIIC protein produced, typically to sporulation. The sporulated cells containing the crystal protein may then be harvested in accordance with conventional methods, e.g., centrifugation or filtration.

The cryIIIC gene may also be incorporated into a plant which is capable of expressing the gene and producing CryIIIC protein, rendering the plant more resistant to insect attack. Genetic engineering of plants with the cryIIIC gene may be accomplished by introducing the desired DNA containing the gene into plant tissues or cells, using DNA molecules of a variety of forms and origins that are well know to those skilled in plant genetic engineering. An example of a

technique for introducing DNA into plant tissue is disclosed in European Patent Application Publication No. 0 289 479, published November 2, 1988, of Monsanto Company.

DNA containing the cryIIIC gene or a modified cryIIIC gene capable of producing the CryIIIC protein may be delivered into the plant cells or tissues directly by infectious plasmids, such as Ti, the plasmid from Agrobacterium tumefaciens, viruses or microorganisms like A. tumefaciens, by the use of lysosomes or liposomes, by microinjection by mechanical methods and by other techniques familiar to those skilled in plant engineering.

Variations may be made in the cryIIIC gene nucleotide base sequences, since the various amino acids forming the protein encoded by the gene usually may be determined by more than one codon, as is well known to those skilled in the art. Moreover, there may be some variations or truncation in the coding region of the cryIIIC nucleotide base sequence which allow expression of the gene and production of functionally equivalent forms of the CryIIIC insecticidal protein. These variations which can be determined without undue experimentation by those of ordinary skill in the art with reference to the present specification are to be considered within the scope of the appended claims, since they are fully equivalent to the specifically claimed subject matter.

The present invention will now be described in more detail with reference to the following specific, non-limiting examples. The

examples relate to work which was actually done based on techniques generally known in the art and using commercially available equipment.

The novel B.t. strain EG4961 was isolated following the procedure described in Example 1.

Example 1

Isolation of B.t. Strain EG4961

Crop dust samples were obtained from various sources throughout the U.S. and abroad, typically grain storage facilities. The crop dust samples were treated by suspending the crop dust in an aqueous buffer and heating the suspension at 60°C for 30 min. to enrich for heat resistant spore forming Bacillus-type bacteria such as B.t. The treated dust suspensions were diluted in aqueous buffer, and the dilutions were spread on agar plates to allow each individual bacterium from the crop dust to grow into a colony on the surface of the agar plate. After growth, a portion of each colony was transferred from the agar plate to a nitrocellulose filter. The filter was treated with NaOH to lyse the colonies and to fix the DNA from each colony onto the filter.

A modified treatment procedure was developed for use with B.t. colonies utilized in the colony hybridization procedure, since standard techniques applicable to E. coli were found to be unworkable with B.t. In the treatment described above, special conditions were required to assure that the B.t. colonies were in a vegetative state of growth, making them susceptible to lysis with NaOH. Accordingly, after a portion of each colony

was transferred to the nitrocellulose filter, the filter was placed colony side up on an agar medium containing 0.5% (w/v) glucose. The transferred colonies were then allowed to grow on the agar-glucose medium for 5 hours at 30°C. Use of 0.5% glucose in the agar medium and the 5-hour, 30°C growth cycle were critical for assuring that the B.t. colonies were in a vegetative state and thus susceptible to lysis.

Despite the opinion expressed by at least one researcher that attempts to use an existing coleopteran toxin gene as a probe to discover a novel gene that was toxic to the southern corn rootworm would be unsuccessful, a cloned coleopteran toxin gene was used as a specific probe to find other novel and rare coleopteran-toxic strains of B.t. from crop dust samples.

A 2.9 kb HindIII DNA restriction fragment containing the cryIIIA gene, formerly known as the cryC gene of B.t. strain EG2158, described in Donovan et al., Mol. Gen. Genet., 214, pp. 365-372 (1988), was used as a probe in colony hybridization procedures.

The 2.9 kb HindIII cryIIIA DNA fragment, containing the entire cryIIIA gene, was radioactively labeled with alpha-P^{32} dATP and Klenow enzyme, by standard methods. The nitrocellulose filters containing the DNA from ach lysed colony were incubated at 65°C for 16 hours in a buffered solution that contained the radioactively labeled 2.9 kb HindIII cryIIIA DNA probe to hybridize the DNA from the colonies with the DNA from the radioactively labeled cryIIIA

probe. The 65°C hybridization temperature was used to assure that the cryIIIA DNA probe would hybridize only to DNA from colonies that contained a gene that was similar to the cryIIIA DNA probe.

The 2.9 kb cryIIIA probe hybridized to many B.t. colonies from various samples of crop dust. Examination of these colonies revealed, unexpectedly, that they did not contain any cryIII-type genes. These colonies did contain cryI-type genes. The cryI-type genes encode lepidopteran-toxic, coleopteran-nontoxic crystal proteins with molecular masses of approximately 130 kDa. Computer-assisted comparisons of the sequence of the cryIIIA gene with the sequence of several cryI-type genes revealed that the 3'-end of the cryIIIA gene was partially homologous with portions of the cryI-type genes. This finding supported the belief that the 3'-end of the cryIIIA gene was causing the 2.9 kb cryIIIA probe to hybridize to B.t. colonies containing cryI-type genes.

To correct this problem, the 2.9 kb HindIII cryIIIA probe was digested with the enzyme XbaI and a 2.0 kb HindIII-XbaI fragment was purified that contained the cryIIIA gene minus its 3'-end. The 2.0 kb HindIII-XbaI fragment contains the 3'-truncated cryIIIA gene. When the 2.0 kb fragment was used in repeated colony hybridization experiments, it did not hybridize to cryI gene-containing B.t. colonies.

Approximately 48,000 Bacillus-type colonies from crop dust samples from various locations were probed with the radioactively labeled 2.0 kb HindIII-XbaI cryIIIA probe. Only

one novel B.t. strain from an Illinois crop dust sample was discovered that specifically hybridized to the cryIIIA probe. That novel strain was designated B.t. strain EG2838, which has been deposited with the NRRL under Accession No. NRRL B-18603.

Subsequently, an additional 50,000 Bacillus-type colonies from crop dust samples were also screened with the radioactively labeled 2.0 kb HindIII-XbaI cryIIIA probe, but without success in identifying any other strains containing novel cryIII-type genes.

B.t. strain EG2838 was found to be insecticidally active against coleopteran insects, notably, the Colorado potato beetle. B.t. strain EG2838 did not have substantial insecticidal activity with respect to the southern corn rootworm. A gene, designated the cryIIIB gene, was isolated from B.t. strain EG2838, and its nucleotide base squence determined. The cryIIIB encoded a crystal protein, designated the CryIIIB protein, containing 651 amino acids having a deduced size of 74,237 Daltons. The size of the prior art CryIIIA protein had previously been deduced to be 73,116 Daltons (644 amino acids). The cryIIIB gene is 75% homologous with the cryIIIA gene, and the CryIIIB protein is 68% homologous with the CryIIIA protein.

Approximately 40,000 Bacillus-type colonies from thirty-nine crop dust samples from various locations from around the world were screened with a cryIIIB probe obtained from B.t. strain EG2838. The cryIIIB probe was radioactively

labeled using the procedure set forth above with respect to the radioactively labeled cryIIIA probe. The radioactively labeled cryIIIB probe consisted of a 2.4 kb SspI restriction fragment of DNA from B.t. strain EG2838. The fragment contains the complete protein coding region for the coleopteran toxin cryIIIB gene of B.t. strain EG2838. Ultimately, a novel B.t. strain from a crop dust sample was discovered that specifically hybridized to the cryIIIB probe. The strain was designated B.t. strain EG4961.

To characterize B.t. strain EG4961, several studies were conducted. One series of studies was performed to characterize its flagellar serotype. Additional studies were conducted to determine the sizes of the native plasmids in B.t. strain EG4961 and to ascertain which plasmids contained genes that encode insecticidal crystal proteins. DNA blot analysis was performed to determine whether any of the native plasmids of B.t. strain EG4961 hybridized with the cryIIIB probe. Also of interest was whether the cryIIIB-hybridizing DNA element of B.t. strain EG4961 was carried on a single naturally occurring plasmid, as opposed to being carried on multiple plasmids or on the chromosomal DNA. In addition, B.t. strain EG4961 was evaluated further by characterizing the crystal proteins it produced and by measuring the insecticidal activity associated with B.t. strain EG4961 and its crystal proteins. Examples 2 through 6 are directed to the procedures for

characterizing B.t. strain EG4961, and Examples 8 through 12 are directed to the insecticidal activity of B.t. strain EG4961.

Example 2

Characterization of the Flagellar Serotype of B.t. Strain EG4961

A panel of B.t. type-strain flagellar antibody reagents was constructed for use in serotyping investigations, using B.t. type-strains that are publicly available. B.t. type-strains HD1 (kurstaki, serotype 3ab), HD2 (thuringiensis, serotype 1), HD5 (kenyae, serotype 4ac), HD11 (aizawai, serotype 7), HD12 (morrisoni, serotype 8ab) and HD13 (tolworthi, serotype 9) were grown in liquid cultures (no shaking) under conditions that produce motile, vegetative cells. Flagellar filaments were sheared from the cells by vortexing, cells were removed by centrifugation, and flagellar filaments were collected from the supernatants on 0.2 μm pore size filters. Purified flagellar filament preparations were analyzed by sodium dodecyl sulfate polyacryamide gel electrophoresis (SDS-PAGE). The SDS-PAGE profiles for these B.t. type-strain flagellar filament preparations showed a major protein band for each of these preparations in the range of 20 to 35 kDa.

These purified flagellar filament preparations were used for antibody production in mice following standard procedures. The resulting antisera were screened for reaction in a standard antibody-mediated cell agglutination assay. In this assay, serial dilutions of antisera were made in a round bottomed 96-well microplate. Formalin-

fixed cell suspensions of B.t. type-strains (or sample strains to be serotyped) were added to the wells and left undisturbed until cell mass was visible near well bottoms. Assays were scored visually for cell agglutination from the bottom of the plate using a magnifying mirror. Antisera giving the strongest specific reaction with cells of the B.t. type-strain from which they were derived were used as flagellar antibody reagents.

Cells from each of B.t. strains EG2158 and EG4961 were separately inputted as samples in a cell agglutination assay using a panel of flagellar antibody reagents from the six B.t. type-strains. Cells of each B.t. type-strain were included as controls. Results of this investigation showed that cells of HD1, HD2, HD5, HD11, HD12 and HD13, B.t. type-strains reacted strongly and specifically with their respective flagellar antibody reagents. B.t. strain EG2158 cells reacted strongly and specifically with the morrisoni (B.t. type-strain HD12) flagellar antibody reagent, but cells from B.t. strain EG4961 did not react with any of the antibody reagents. These results confirm that B.t. strain EG2158 is a subspecies morrisoni B.t. strain and indicate that B.t. strain EG4961 is not a subspecies morrisoni, kurstaki, thuringiensis, kenyae, aizawai or tolworthi.

Example 3

Size Fractionation and cryIIIB Probing of Native Plasmids of EG4961

B.t. strains may be characterized by fractionating their plasmids according to size by the well-known procedure called agarose gel

electrophoresis. The procedure involves lysing B.t. cells with lysozyme and SDS, electrophoresing plasmids from the lysate through an agarose gel and staining the gel with ethidium bromide to visualize the plasmids. Larger plasmids, which move more slowly through the gel, appear at the top of the gel and smaller plasmids appear toward the bottom of the gel.

The agarose gel in Figure 2 shows that B.t. strain EG4961 contains native plasmids of approximately 150, 95, 70, 50, 5 and 1.5 MDa, as indicated by the dark horizontal bands. Plasmid sizes were estimated by comparison to plasmids of known sizes (not shown). Figure 2 further shows that the coleopteran-toxic B.t. strain EG2838 contains native plasmids of about 100, 90 and 37 MDa. Figure 2 also shows that the coleopteran-toxic B.t. strain EG2158 contains native plasmids of about 150, 105, 88, 72, and 35 MDa. Some of the plasmids, such as the 150 and 1.5 MDa plasmids of B.t. strain EG4961 and the 150 MDa plasmid of B.t. strain EG2158, may not be visible in the photograph, although they are visible in the actual gel. Figure 2 demonstrates that the sizes of the native plasmids of B.t. strain EG4961 are different from the sizes of the native plasmids of B.t. strains EG2158 and EG2838.

The plasmids shown in Figure 2 were transferred by blotting from the agarose gel to a nitrocellulose filter using the blot techniques of Southern, J. Molec. Biol., 98, pp. 503-517 (1975), and the filter was hybridized as described above with the radioactively labeled 2.4 kb cryIIIB DNA

probe. After hybridization, the filter was exposed to X-ray film. A photograph of the X-ray film is shown in Figure 3 which shows by the darkened area that the cryIIIB probe hybridized to the 95 MDa plasmid of B.t. strain EG4961. This result demonstrates that the 95 MDa plasmid of B.t. strain EG4961 contains a DNA sequence that is at least partly homologous to the cryIIIB gene. Figure 3 also shows that the cryIIIB probe hybridized, as expected, to the 88 MDa plasmid of B.t. strain EG2158 and to the 100 MDa plasmid of B.t. strain EG2838. The 88 MDa plasmid of B.t. strain EG2158 has been previously shown to contain the coleopteran-toxin cryIIIA gene (see Donovan et al., Mol. Gen. Genet., 214, pp. 365-372 (1988)). It has been determined that the 100 MDa plasmid of B.t. strain EG2838 contains the coleopteran toxin cryIIIB gene.

The cryIIIB probe also hybridized to small bands of DNA in each of B.t. strains EG4961, EG2838 and EG2158 that are indicated by the letter "f" in Figure 3. Previous experience has shown that large B.t. plasmids often break into fragments during electrophoresis. These fragments normally migrate to the position of the bands indicated by the letter "f" in Figure 3. Therefore, the bands indicated by the letter "f" in Figure 3 are most likely derived by fragmentation of the 95 MDa, 88 MDa and 100 MDa plasmids of B.t. strains EG4961, EG2158 and EG2838, respectively.

Example 4

Blot Anaylsis of DNA from B.t. Strain EG4961

Both chromosomal and plasmid DNA from B.t. strain EG4961 was extracted and digested with HindIII plus EcoRI restriction enzymes. The digested DNA was size fractionated by electrophoresis through an agarose gel, and the fragments were visualized by staining with ethidium bromide. Figure 4 is a photograph of the stained agarose gel that contains size fractionated HindIII and EcoRI restriction fragments of B.t. strain EG4961. For comparison, the DNA from the coleopteran-toxic B.t. strains EG2158 and EG2838 was processed in an identical manner. The lane labeled "stnd" contains lambda DNA fragments of known sizes, which serve as size standards. Figure 4 shows that HindIII plus EcoRI digested B.t. DNA yields hundreds of DNA fragments of various sizes.

The DNA shown in Figure 4 was transferred from the agarose gel to a nitrocellulose filter, and the filter was hybridized at 65°C in a buffered aqueous solution containing the radioactively labeled 2.4 kb cryIIIB DNA probe. After hybridization, the filter was exposed to X-ray film. Figure 5 is a photograph of the X-ray film where the numbers to the right indicate the size, in kb, of the cryIIIB hybridizing fragments of B.t. strain EG4961 as determined by comparison with lambda DNA digested with HindIII as a size marker in the lane labeled "stnd". Figure 5 shows that HindIII plus EcoRI digested DNA of B.t. strain EG4961 yields cryIIIB-hybridizing fragments of approximately 3.8 kb and 2.4 kb. Figure 5 also

shows that HindIII plus EcoRI digested DNA of B.t. strain EG2838 yields cryIIIB-hybridizing fragments of approximately 2.9 kb and 3.8 kb. Figure 5 further shows that the approximate sizes of cryIIIB-hybridizing restriction DNA fragments of B.t. strain EG2158 are 1.6 kb and 0.7 kb.

These results suggest that B.t. strain EG4961 contains a cryIII-type gene that is related to the cryIIIB gene probe. The cryIIIB-hybridizing fragments of B.t. strain EG4961 are different from those of B.t. strains EG2838 and EG2158. These results and further studies described in the Examples below confirm that the cryIII-type gene of B.t. strain EG4961 is clearly different from the cryIIIB gene of EG2838 and the cryIIIA gene of EG2158. The cryIII-type gene of B.t. strain EG4961 has been designated cryIIIC.

Example 5

Characterization of Crystal Proteins of B.t. Strain EG4961

B.t. strain EG4961 was grown in DSMG sporulation medium at 30°C until sporulation and cell lysis had occurred (3 to 4 days growth). The DSMG medium is 0.4% (w/v) Difco nutrient broth, 25 mM K_2HPO_4, 25 mM KH_2PO_4, 0.5 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$, 10 µM $FeSO_4$, 10 µM $MnCl_2$ and 0.5% (w/v) glucose. The sporulated culture of B.t. strain EG4961 was observed microscopically to contain free floating, irregularly shaped crystals in addition to B.t. spores. Experience has shown that B.t. crystals are usually composed of proteins that may be toxic to specific insects. The appearance of the crystals of B.t. strain EG4961 differed from

the flat, rectangular (or rhomboidal) crystals of B.t. strain EG2158, but partially resembled some of the irregularly shaped crystals of B.t. strain EG2838.

Spores, crystals and residual lysed cell debris from the sporulated culture of B.t. strain EG4961 were harvested by centrifugation. The crystals were specifically solubilized from the centrifuged fermentation culture solids (containing crystals, spores and some cell debris) by heating the solids mixture in a solubilization buffer (0.13 M Tris pH 8.5, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol) at 100°C for 5 min. The solubilized crystal proteins were size fractionated by SDS-PAGE. After size fractionation, the proteins were visualized by staining with Coomassie dye. Cultures of B.t. strains EG2158 and EG2838 are processed in an identical manner for purposes of comparison.

Figure 6 shows the results of these analyses where the numbers to the right indicate the size, in kDa, of the crystal proteins synthesized by B.t. strain EG4961. A major protein of approximately 70 kDa and a minor protein of approximately 30 kDa were solubilized from centrifuged fermentation solids containing B.t. strain EG4961 spores and crystals. The approximately 70 kDa protein of B.t. strain EG4961 appears similar in size to the approximately 70 kDa coleopteran-toxic crystal protein of B.t. strain EG2158 and to the approximately 70 kDa coleopteran-toxic crystal protein of B.t. strain EG2838. The minor crystal protein of approximately

30 kDa of B.t. strain EG4961 is roughly similar in size to crystal proteins of approximately 31 kDa and 29 kDa produced by B.t. strain EG2158 and to crystal proteins of approximately 28 kDa and 32 kDa produced by B.t. strain EG2838. It is not known whether these small proteins are related to one another.

Following the procedure of Example 4, further DNA blot analysis revealed that the 2.4 kb cryIIIB DNA probe specifically hybridized to a single 8.3 kb Asp718-PstI restriction fragment of B.t. strain EG4961 DNA. This result suggested that the 8.3 kb fragment contained the complete cryIIIC gene.

The 8.3 kb Asp718-PstI fragment of B.t. strain EG4961 was isolated and studies were conducted on the 8.3 kb Asp718-PstI restriction fragment to confirm that the fragment contained a cryIII-type gene and to identify and determine the nucleotide base sequence of the cryIIIC gene. The procedures are set forth in Example 6.

Example 6

Cloning and Sequencing of the cryIIIC Gene of B.t. Strain EG4961

To clone the 8.3 kb fragment described in the previous Example, a plasmid library of B.t. strain EG4961 was constructed by ligating size-selected DNA Asp718-PstI restriction fragments from B.t. strain EG4961 into the well-known E. coli vector pUC18. This procedure involved first obtaining total DNA from B.t. strain EG4961 by cell lysis followed by spooling, then double digesting the total DNA with both Asp718 and PstI restriction

enzymes, electrophoresing the digested DNA through an agarose gel, excising a gel slice containing 7 kb-9 kb size selected fragments of DNA, and electroeluting the size selected Asp718-PstI restriction fragments from the agarose gel slice. The selected fragments were mixed with the E. coli plasmid vector pUC18, which had also been digested with Asp718 and PstI. The pUC18 vector carries the gene for ampicillin resistance (Amp^r) and the vector replicates in E. coli. T4 DNA ligase and ATP were added to the mixture of size-selected restriction fragments of DNA from B.t. strain EG4961 and of digested pUC18 vector to allow the pUC18 vector to ligate with the B.t. strain EG4961 restriction fragments.

The plasmid library was then transformed into E. coli cells, a host organism lacking the gene of interest, as follows. After ligation, the DNA mixture was incubated with an ampicillin sensitive E. coli host strain, E. coli strain HB101, that had been treated with $CaCl_2$ to allow the cells to take up the DNA. E. coli, specifically strain HB101, was used as the host strain because these cells are easily transformed with recombinant plasmids and because E. coli strain HB101 does not naturally contain genes for B.t. crystal proteins. Since pUC18 confers resistance to ampicillin, all host cells acquiring a recombinant plasmid would become ampicillin-resistant. After exposure to the recombinant plasmids, the E. coli host cells were spread on agar medium that contained ampicillin. Several thousand E. coli colonies grew on the ampicillin-

containing agar from those cells which harbored a recombinant plasmid. These E. coli colonies were then blotted onto nitrocellulose filters for subsequent probing.

The radioactively labeled 2.4 kb cryIIIB gene probe was then used as a DNA probe under conditions that permitted the probe to bind specifically to those transformed host colonies that contained the 8.3 kb Asp718-PstI fragment of DNA from B.t. strain EG4961. Twelve E. coli colonies specifically hybridized to the 2.4 kb cryIIIB probe. One cryIIIB-hybridizing colony, designated E. coli strain EG7218, was studied further. E. coli strain EG7218 contained a recombinant plasmid, designated pEG258, which consisted of pUC18 plus the 8.3 kb Asp718-PstI restriction fragment of DNA. The cryIIIB probe specifically hybridized to the 8.3 kb fragment of pEG258. A restriction map of pEG258 is shown in Figure 7.

The 8.3 kb fragment of pEG258 contained HindIII fragments of 2.4 kb and 3.8 kb, and a BamHI-XbaI fragment of 4.0 kb that specifically hybridized with the cryIIIB probe. The 2.4 kb HindIII fragment was subcloned into the DNA sequencing vector M13mp18. The 4.0 kb BamHI-XbaI fragment was subcloned into the DNA sequencing vectors M13mp18 and M13mp19.

The nucleotide base sequence of a substantial part of each subcloned DNA fragment was determined using the standard Sanger dideoxy method. For each subcloned fragment, both DNA strands were sequenced by using sequence-specific

17-mer oligonucleotide primers to initiate the DNA sequencing reactions. Sequencing revealed that the 8.3 kb fragment contained an open reading frame and, in particular, a new cryIII-type gene. This new gene, designated cryIIIC, is significantly different from the cryIIIA gene. As indicated below, cryIIIC gene is also clearly distinct from the cryIIIB gene.

The DNA sequence of the cryIIIC gene and the deduced amino acid sequence of the CryIIIC protein encoded by the cryIIIC gene are shown in Figure 1. The protein coding portion of the cryIIIC gene is defined by the nucleotides starting at position 14 and ending at position 1972. The probable ribosome binding site is indicated as "RBS" in Figure 1-1. The size of the CryIIIC protein encoded by the cryIIIC gene, as deduced from the open reading frame of the cryIIIC gene, is 74,393 Daltons (651 amino acids). It should be noted that the apparent size of the CryIIIC protein, as determined from SDS-PAGE, is approximately 70 kDa. Therefore, the CryIIIC protein will be referred to in this specification as being approximately 70 kDa in size.

The size of the prior art CryIIIA protein has previously been deduced to be 73,116 Daltons (644 amino acids). The size of the CryIIIB protein has previously been determined to be 74,237 Daltons (651 amino acids).

DNA sequencing revealed the presence of BamHI and HindIII restriction sites within the cryIIIC gene (See Figure 1-2). Knowledge of the locations of these restriction sites permitted the

precise determination of the location and orientation of the cryIIIC gene within the 8.3 kb fragment as indicated by the arrow in Figure 7.

The computer program of Queen and Korn (C. Queen and L.J. Korn, "Analysis of Biological Sequences on Small Computers," DNA, 3, pp. 421-436 (1984)) was used to compare the sequences of the cryIIIC gene to the cryIIIB and cryIIIA genes and to compare the deduced amino acid sequences of their respective CryIIIC, CryIIIB and CryIIIA proteins.

The nucleotide base sequence of the cryIIIC gene was 96% positionally identical with the nucleotide base sequence of the cryIIIB gene and only 75% positionally identical with the nucleotide base sequence of the cryIIIA gene. Thus, although the cryIIIC gene is related to the cryIIIB and cryIIIA genes, it is clear that the cryIIIC gene is distinct from the cryIIIB gene and substantially different from the cryIIIA gene.

The deduced amino acid sequence of the CryIIIC protein was found to be 94% positionally identical to the deduced amino acid sequence of the CryIIIB protein, but only 69% positionally identical to the deduced amino acid sequence of the CryIIIA protein. These differences, together with the differences in insecticidal activity as set forth below, clearly show that the CryIIIC protein encoded by the cryIIIC gene is a different protein from the CryIIIB protein or the CryIIIA protein.

Moreover, while not wishing to be bound by any theory, based on a comparison of the amino acid sequences of the CryIIIC protein and the

CryIIIB protein, it is believed that the following amino acid residues may be of significance for the enhanced corn rootworm toxicity of the CryIIIC protein, where the numbers following the accepted abbreviations for the amino acids indicate the position of the amino acid in the sequence illustrated in Figure 1: His9, His231, Gln339, Phe352, Asn446, His449, Val450, Ser451, Lys600 and Lys624. These amino acid residues were selected as being of probable significance for the corn rootworm toxicity of the CryIIIC protein because, after studying the amino acid sequences of several other CryIII proteins, the amino acids at the indicated positions fairly consistently showed different amino acids than those indicated for the CryIIIC protein.

Example 7

Expression of the Cloned cryIIIC Gene

Studies were conducted to determine the production of the CryIIIC protein by the cryIIIC gene.

Table 1 summarizes the relevant characteristics of the B.t. and E. coli strains and plasmids used during these procedures. A plus ($^+$) indicates the presence of the designated element, activity or function and a minus ($^-$) indicates the absence of the same. The designations ^{s} and ^{r} indicate sensitivity and resistance, respectively, to the antiobiotic with which each is used. The abbreviations used in the table have the following meanings: Amp (ampicillin); Cm (chloramphenicol); Cry (crystalliferous); Tc (tetracycline).

Table 1

Strains and Plasmids

Strain or plasmid	Relevant characteristics
B. thuringiensis	
HD73-26	Cry^-, Cm^S
EG7211	HD73-26 harboring pEG220(Cry^-)
EG7220	HD73-26 harboring pEG260(cryIIIC$^+$ cryX$^+$)
EG7231	HD73-26 harboring pEG269(cryIIIC$^+$ cryX$^-$)
EG4961	cryIIIC$^+$ cryX$^+$
E. coli	
DH5α	Cry^-, Amp^S
GM2163	Cry^-, Amp^S
EG7218	DH5α harboring pEG258(cryIIIC$^+$ cryX$^+$)
EG7221	DH5α harboring pUC18(Cry^-)
EG7232	DH5α harboring pEG268(cryIIIC$^+$ cryX$^-$)
EG7233	DH5α harboring pEG269(cryIIIC$^+$ cryX$^-$)
Plasmids	
pEG220	Amp^r, Tc^r, Cm^r, Cry^-, Bacillus-E. coli shuttle vector consisting of pBR322 ligated into the SphI site of pNN101
pUC18	Amp^r, Cry^-, E. coli vector
pNN101	Cm^r, Tc^r, Cry^-, Bacillus vector
pEG258	Amp^r, cryIIIC$^+$ cryX$^+$ E. coli recombinant plasmid consisting of the 8.3 kb Asp718-PstI cryIIIC$^+$ cryX$^+$ fragment of B.t. strain EG4961 ligated into the Asp718-PstI sites of pUC18

Table 1 (continued)

Strains and Plasmids

Strain or plasmid	Relevant characteristics
Plasmids (continued)	
pEG260	Tc^r, Cm^r, *cryIIIC*$^+$ *cryX*$^+$ *Bacillus* recombinant plasmid consisting of the 8.3 kb *Asp*718-*Pst*I *cryIIIC*$^+$ *cryX*$^+$ fragment of *B.t.* strain EG4961 blunt ligated into the *Eco*RV site of pNN101
pEG268	Amp^r *cryIIIC*$^+$ *cryX*$^-$ *E. coli* recombinant plasmid consisting of a 5 kb *Sau*3A fragment of *B.t.* strain EG4961 ligated into the *Bam*HI site of pBR322
pEG269	Amp^r (*E. coli*), Tc^r and Cm^r (*B.t*), *cryIIIC*$^+$ *cryX*$^-$, recombinant shuttle plasmid consisting of pNN101 ligated into the *Sph*I site of pEG268

E. coli cells harboring the cloned 8.3 kb fragment described in Example 6 were analyzed to determine if they produced the 70 kDa CryIIIC crystal protein.

Experience has shown that cloned *B.t.* crystal genes are poorly expressed in *E. coli* and highly expressed in *B.t.* Recombinant plasmid pEG258, constructed as set forth in Example 6, will replicate in *E. coli*, but not in *B.t.* To achieve a high level of expression of the cloned *cryIIIC* gene, the 8.3 kb *cryIIIC* fragment was transferred from pEG258 to a plasmid vector pNN101 (Tc^r Cm^r Cry^-) that is capable of replicating in *B.t.*

The plasmid construct pEG258 was isolated from E. coli strain EG7218 by lysozyme/SDS treatment, followed by ethanol precipitation of the plasmid DNA, all using standard procedures. The pEG258 plasmid DNA was then used to transform cells of E. coli strain GM2163 made competent by the calcium chloride procedure described earlier in Example 6. E. coli strain GM2163 is a crystal negative (Cry^-) and ampicillin sensitive (Amp^S) strain, constructed by the procedures of M.G. Marinus et al. in Mol. Gen. Genet., 192, pp. 288-289 (1983).

The plasmid construct pEG258 was again isolated, this time from the transformed E. coli strain GM 2163, using the procedures just described. The isolated pEG258 plasmid DNA was digested with Asp718 and PstI. The digested plasmid was electrophoresed through an agarose gel and the 8.3 kb Asp718-PstI cryIIIC fragment was electroeluted from the agarose gel. The 8.3 kb fragment was made blunt-ended by using T4 polymerase and deoxynucleotide triphosphates to fill in the Asp718 and PstI ends.

The blunt-ended 8.3 kb fragment was mixed with the Bacillus vector pNN101 that had been digested with EcoRV. T4 DNA ligase and ATP were added to the mixture to allow the blunt-ended 8.3 kb fragment to ligate into the EcoRV site of the pNN101 vector. After ligation, the DNA mixture was added to a suspension of B.t. strain HD73-26 cells. Cells of B.t. strain HD73-26 are crystal-negative (Cry^-) and chloramphenicol sensitive (Cm^S). Using electroporation techniques, the cells

of B.t. strain HD73-26 in the mixture were induced to take up the recombinant plasmid construct, consisting of pNN101 and the ligated 8.3 kb cryIIIC fragment, also present in the mixture. Thus, the recombinant plasmid was transformed by electroporation into B.t. strain HD73-26.

After electroporation, the transformed B.t. cells were spread onto an agar medium containing 5 µg chloramphenicol and were incubated about 16-18 hours at 30°C. Cells that had taken up the plasmid pNN101 would grow into colonies on the chloramphenicol agar medium whereas cells that had not absorbed the plasmid would not grow. Cm^r colonies were transferred onto nitrocellulose and then probed with the radioactively labeled cryIIIB gene and one colony, designated B.t. strain EG7220, that specifically hybridized to the cryIIIB probe was studied further.

EG7220 contained a plasmid, designated pEG260, that consisted of the 8.3 kb cryIIIC fragment inserted into the EcoRV site of the pNN101 vector. A restriction map of plasmid pEG260 is shown in Figure 8.

Cells of B.t. strain EG7220 were grown in a sporulation medium containing chloramphenicol (5 µg/ml) at 23-25°C until sporulation and cell lysis had occurred (3-4 days). Microscopic examination revealed that the culture of B.t. strain EG7220 contained spores and free floating irregularly shaped crystals.

Spores, crystals and cell debris from the sporulated fermentation culture of B.t. strain EG7220 were harvested by centrifugation. The

crystals were solubilized by heating the centrifuged fermentation solids mixture in solubilization buffer (0.13 M Tris pH 8.5, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol) at 100°C for 5 min. After heating, the mixture was applied to an SDS-polyacryamide gel and proteins in the mixture were size fractionated by electrophoresis. After size fractionization, the proteins were visualized by staining with Coomassie dye. A photograph of the Coomassie stained gel is shown in Figure 10.

Figure 10 shows that B.t. strain EG7220 produced a major protein of approximately 70 kDa and a minor protein of approximately 30 kDa. These proteins appeared to be identical in size with the major approximately 70 kDa protein and the minor approximately 30 kDa protein produced by B.t. strain EG4961 (Figure 10). This result demonstrates that the 8.3 kb fragment of pEG260 contains two crystal protein genes: one for the approximately 70 kDa protein and one for the approximately 30 kDa protein.

The gene encoding the approximately 70 kDa protein is the cryIIIC gene, and the encoded protein is the CryIIIC protein. The gene encoding the approximately 30 kDa crystal protein has been designated cryX, and the encoded protein has been designated CryX.

As expected and as illustrated in Figure 10, an isogenic control strain of B.t., designated EG7211, consisting of B.t. strain HD73-26 and harboring only the plasmid vector pEG220, did not produce the approximately 70 kDa protein or the

approximately 30 kDa protein. Plasmid pEG220 is an ampicillin resistant, tetracycline resistant, chloramphenicol resistant and crystal-negative E. coli-Bacillus shuttle vector consisting of pBR322 ligated into the SphI site of pNN101.

E. coli cells harboring the cloned 8.3 kb fragment containing the cryIIIC gene and the cryX gene were analyzed to determine whether they produced the approximately 70 kDa and approximately 30 kDa crystal proteins. E. coli cells harboring pEG258, designated strain EG7218, were grown to late stationary phase and cells were harvested by centrifugation. E. coli strain EG7218 cells were lysed and total cellular proteins were solubilized by heating the cells in the protein buffer. The complement of proteins solubilized from E. coli EG7218 cells appeared identical to the complement of proteins solubilized from a negative control strain of E. coli, designated EG7221, that harbored only the plasmid vector pUC18 as illustrated in Figure 10. This result demonstrates that E. coli cells harboring the cloned 8.3 kb cryIIIC fragment produce very little, if any, of either the approximately 70 kDa or the approximately 30 kDa crystal proteins.

The following procedures were used to isolate the cryIIIC gene, responsible for making the approximately 70 kDa CryIIIC protein.

A Sau3A fragment of DNA from B.t. strain EG4961 that contained the cryIIIC gene, but not the cryX gene, was cloned by using the cryIIIB gene as a probe. This was accomplished by partially digesting DNA from B.t. strain EG4961 with Sau3A,

electrophoresing the digested DNA through an agarose gel and excising a gel slice containing Sau3A fragments of 4 kb to 9 kb. The Sau3A fragments were electroeluted from the gel slice and mixed with plasmid pBR322 vector that had been digested with BamHI. The Sau3A fragments were ligated with the pBR322 vector. The ligation mix was incubated with $CaCl_2$-treated cells of E. coli strain DH5α to allow the cells to take up plasmid DNA.

After incubation, the cells were plated on agar plates containing ampicillin and LB medium (1% (w/v) Difco tryptone, 0.5% (w/v) Difco yeast extract, 0.5% (w/v) NaCl, pH 7.0), to select for those cells that had absorbed plasmid DNA. Several hundred Amp^r transformant colonies were blotted onto nitrocellulose filters and the filters were probed with the radioactively labeled cryIIIB probe as described above in Example 1. The probe hybridized to several colonies and the characterization of one of these colonies, designated EG7232, is further described here. E. coli strain EG7232 contained a plasmid, designated pEG268, that consisted of pBR322 plus an inserted Sau3A-BamHI DNA fragment of approximately 5 kb. The inserted DNA fragment specifically hybridized to the radioactively labeled cryIIIB probe.

Plasmid pEG268 (Amp^r Tc^s) will replicate in E. coli but not in B.t. To obtain a derivative of pEG268 that could replicate in B.t., pEG268 was digested with SphI, mixed with the Bacillus plasmid pNN101 (CM^r Tc^r) that had also been digested with SphI and the mixture was ligated. The ligation

mixture was incubated with a suspension of $CaCl_2$-treated E. coli cells to allow the cells to take up DNA from the pEG268 plasmid ligated with pNN101. After incubation, the cells were plated on agar plates containing LB medium and tetracycline, and several hundred tetracycline resistant colonies grew. Only those cells that had absorbed a plasmid consisting of pEG268 and pNN101 would be able to grow and form colonies in the presence of tetracycline. The characterization of one of these Tc^r colonies, designated EG7233, was selected for further study. As expected, E. coli strain EG7233 was found to contain a plasmid, designated pEG269, that consisted of pNN101 inserted into the SphI site of pEG268. A restriction map of pEG269 is shown in Figure 9.

The plasmid construct pEG269 was isolated from E. coli strain EG7233 by lysozyme/SDS treatment, followed by ethanol precipitation of the plasmid DNA, all using standard procedures. The pEG269 plasmid DNA was then used to transform cells of E. coli strain GM2163 made competent by the calcium chloride procedure, all as described earlier.

The plasmid construct pEG269 was again isolated, this time from the transformed E. coli strain GM2163. The isolated pEG269 plasmid DNA was added to a suspension of cells of the crystal-negative, chloramphenicol-sensitive B.t. strain HD73-26 and an electric current was passed through the mixture, such that pEG269 was transformed by electroporation into B.t. strain HD73-26. The cells were plated onto an agar plate containing LB

medium and chloramphenicol and, after incubation, several hundred Cm^r colonies grew. The characterization of one of these Cm^r colonies, designated EG7231, was selected for investigation. As expected, B.t. strain EG7231 was found to contain pEG269.

Cells of B.t. strain EG7231 were grown in DSMG medium containing chloramphenicol at 20-23°C for 4 days. Microscopic examination showed that the culture contained, in addition to spores, particles that resembled B.t. crystals. The culture solids including spores, crystals and cell debris were harvested by centrifugation and suspended in an aqueous solution at a concentration of 100 mg of culture solids/ml. A portion of this suspension was mixed with solubilization buffer (0.13 M Tris pH 8.5, 2% w/v SDS, 5% v/v 2-mercapto-ethanol, 10% v/v glycerol), heated at 100°C for 5 minutes and the mixture was electrophoresed through an SDS-polyacrylamide gel to size fractionate proteins. After size fractionation, the proteins were visualized by staining the gel with Coomassie dye. A photograph of the stained gel is included in Figure 10.

B.t. strain EG7231 produced a major protein of approximately 70 kDa that appeared to be identical in size to the approximately 70 kDa CryIIIC protein produced by B.t. strain EG4961, as indicated in Figure 10. B.t. strain EG7231 did not produce any detectable amount of the approximately 30 kDa crystal protein (Figure 10). This result demonstrates that the cryX gene for the approximately 30 kDa crystal protein is located

within the region indicated by the dotted line in Figures 7 and 8. Furthermore, this shows that B.t. strain EG7231 contains the cryIIIC gene in isolated form.

The following Examples 8-12 describe the manner in which the insecticidal activity of B.t. strain EG4961 and of the CryIIIC protein was determined.

Insecticidal Activity of B.t. Strain EG4961 and the CryIIIC Protein Compared to B.t. Strain EG2158, B.t. tenebrionis and the CryIIIA Protein

Example 8

General Preparation and Testing Procedures for Insecticidal Bioassays

Fermentation concentrates. B.t. strains EG4961 and EG2158 and B.t. tenebrionis ("B.t.t.") were grown in a liquid sporulation medium at 30°C until sporulation and lysis had occurred. The medium contained a protein source, a carbohydrate source, and mineral salts and is typical of those in the art. NaOH was added to adjust the medium to pH 7.5 prior to autoclaving. The fermentation broth was concentrated by centrifugation and refrigerated until use.

As used herein, "CryIII" crystal protein designates the crystal protein of approximately 70 kDa obtained from the cultures of each of B.t. strains EG4961 and EG2158 and B.t.t. being tested. The CryIII crystal proteins were purified from the

fermentation culture solids using sucrose density gradients. When using sucrose density gradients to separate the components of the fermentation culture of sporulated B.t., B.t. spores form a pellet at the bottom of the gradient and B.t. crystals form a band at approximately the middle of the gradient. Thus, sucrose density gradients permit the separation of B.t. crystal proteins, in relatively pure form, from B.t. spores and other fermentation culture solids. The separated CryIII crystal proteins were stored at 4°C until use.

Quantification of the amount of CryIII crystal protein in all samples bioassayed was determined using standard SDS-PAGE techniques. The following insects were tested:

southern corn rootworm (SCRW)	Diabrotica undecimpunctata howardi
western corn rootworm (WCRW)	Diabrotica virgifera virgifera
Colorado potato beetle (CPB)	Leptinotarsa decemlineata
elm leaf beetle	Pyrrhalta luteola
imported willow leaf beetle	Plagiodera versicolora

Two types of bioassays were performed, one using an artificial diet and the other using a leaf dip.

Artificial diet bioassays. SCRW larvae were bioassayed via surface contamination of an artificial diet similar to Marrone et al., J. Econ. Entomol., 78, pp. 290-293 (1985), but without formalin. Each bioassay consisted of eight serial aqueous dilutions with aliquots applied to the surface of the diet. After the diluent (an aqueous 0.005% Triton® X-100 solution) had dried, first

instar larvae were placed on the diet and incubated at 28°C. Thirty-two larvae were tested per dose. Mortality was scored after 7 days. Data from replicated bioassays were pooled for probit analysis (R.J. Daum, Bull. Entomol. Soc. Am., 16, pp. 10-15 (1970)) with mortality corrected for control death, the control being the diluent only (W.S. Abbott, J. Econ. Entomol., 18, pp. 265-267 (1925)). Results are reported by amount of CryIII crystal protein per mm^2 of diet surface resulting in LC_{50}, the concentration killing 50% of the test insects. 95% confidence intervals are reported within parentheses.

First instar WCRW larvae were tested on the same artificial diet at one dose. Mortality was read at 48 hours.

First instar CPB larvae were tested using similar techniques, except for the substitution of BioServe's #9380 insect diet with potato flakes added for the artificial diet. Mortality was scored at three days instead of seven days.

Leaf dip bioassays. For insect species or stages where suitable artificial diets were not available, bioassays were conducted by dipping suitable natural food materials (leaves) into known treatment concentrations suspended in an aqueous 0.2% Triton® X-100 solution. After excess material had dripped off, the leaves were allowed to dry. Leaves dipped in 0.2% Triton® X-100 served as untreated controls. Five or ten insects were confined in a petri dish with a treated leaf and allowed to feed for 48 hours. SCRW adults, CPB adults, elm leaf beetle larvae and adults, and

imported willow leaf beetle larvae and adults were tested in this manner using appropriate food sources.

Any deviations from the above methodologies are noted with the appropriate data.

Example 9

Insecticidal activity of CryIII proteins against CPB larvae, elm leaf beetles and imported willow leaf beetle larvae

B.t. strain EG4961 is similar in activity to the previously discovered B.t. strain EG2158 against CPB larvae when tested on artificial diet, as shown by the data in Table 2.

Table 2

Insecticidal activity of B.t. strains EG4961 and EG2158 against first instar Colorado potato beetle larvae in artificial diet bioassays

		LC_{50} (95% C.I.)* in ng CryIII/mm^2	
Sample Type	Assays	EG4961	EG2158
Ferm. conc.	2	0.47 (0.39-0.57)	0.42 (0.35-0.50)
Control mortality		3.1%	

95% confidence interval set forth in parentheses

Leaf dip bioassays have also demonstrated that B.t. strain EG4961 is similar in activity to B.t. strain EG2158 and B.t.t. against elm leaf beetle larvae and adults and imported willow leaf beetle larvae.

Example 10

Insecticidal activity of B.t. strains and CryIII Proteins against SCRW larvae in artificial diet bioassays

B.t. strain EG4961 possesses unique activity against SCRW larvae compared to B.t. strain EG2158 and B.t.t. in artificial diet bioassays, as shown by the bioassay data in Table 3. The comparisons in Table 3 labeled "Ferm. conc. #1" and "Ferm. conc. #2" were based on different fermentation concentrates of B.t. strain EG4961. Neither B.t. strain EG2158 nor B.t.t. caused over 15% mortality at the highest dose tested. In contrast, LC_{50} values (i.e., 50% mortality at the specified dose) were obtained for B.t. strain EG4961 (Table 3).

When the purified CryIIIC crystal protein of B.t. strain EG4961 was bioassayed, the activity observed was only slightly less than that obtained with B.t. strain EG4961 fermentation concentrates (containing spores and crystals). This result identified the CryIIIC crystal protein as the toxic agent in B.t. strain EG4961. Surviving larvae in the B.t. strain EG4961 bioassays (both fermentation concentrates and purified crystal protein) were extremely stunted in growth compared to the untreated control larvae.

What little activity the fermentation concentrate of B.t. strain EG2158 had against SCRW larvae was lost when its purified CryIIIA crystal protein was assayed alone. Even with the concentration of purified CryIIIA protein increased five-fold over the corresponding amount of CryIIIC crystal protein, SCRW activity was non-existent for

the CryIIIA protein. The minimal activity of B.t. strain EG2158 as a fermentation concentrate may have been dependent on the presence of spores along with the CryIIIA crystal protein.

Table 3

Insecticidal activity of B.t. strain EG4961 against SCRW larvae in artificial diet bioassays

	#	LC_{50} (95% C.I.) in ng CryIII/mm^2		
Sample type	assays	EG4961	EG2158	B.t.t.
Ferm. conc. #1	4	170 (139-213)	14% dead @ 1000	not tested
Control mortality #1		9.4%		
Ferm. conc. #2	4	206 (161-273)	not tested	15% dead @ 1000
Control mortality #2		8.6%		
Purified protein crystals	4	645 (521-819)	3% dead @ 5000	not tested
Control mortality		8.3%		

An artificial diet bioassay testing B.t. strain EG4961 fermentation concentrate at one dose against WCRW larvae yielded mortality similar to that observed with SCRW larvae. As with SCRW larvae, B.t.t. yielded little mortality greater than the control.

Example 11

Insecticidal activity of B.t. strains EG4961, EG2158 and B.t.t. against adult SCRW and adult CPB in leaf dip bioassays

In addition to its unique activity against SCRW larvae, B.t. strain EG4961 also exhibits unique insecticidal activity to adult stages of both SCRW and CPB (Table 4) which are relatively unaffected by B.t. strain EG2158 or B.t.t. Insect bioassay data from these studies are shown in Table 4.

Table 4

Insecticidal activity of B.t. strains EG4961, EG2158 and B.t.t. against adult SCRW and adult CPB in leaf dip bioassays

		% dead at 48 hrs.	
Strain	µg CryIII/ml	SCRW	CPB
EG4961	2800	50	100
	1400	37.5	98
	700	25	95
	350	10	70
EG2158	4350	-	-
	2175	-	0
	1088	-	10
	544	-	-
B.t.t.	2250	0	0
	1125	10	5
	563	0	0
Control mortality		0	0

(-) dashes indicate not tested.

Example 12

Insecticidal activity of the cloned cryIIIC gene

B.t. strain EG4961 and recombinant B.t. strain EG7231, containing the cloned cryIIIC gene from B.t. strain EG4961 and described in Example 7, were grown on liquid sporulation medium and concentrated via centrifugation as described generally in Examples 5 through 7. Both concentrates were bioassayed against SCRW larvae and CPB larvae on artificial diet using previously described techniques but with three doses instead of eight and (for CPB) 16 CPB larvae per dose instead of 32. The results set forth in Table 5 demonstrate that B.t. strain EG7231 produces a CryIIIC crystal protein equal in toxicity to that found in B.t. strain EG4961. The crystal negative, sporulating B.t. strain EG7211 used to create B.t. strain EG7231 was tested as an additional control and was not active. This bioassay verifies that the cryIIIC gene produces the coleopteran-active crystal protein in B.t. strain EG4961.

Table 5

Activity of B.t. strains EG7231 and EG4961 against SCRW larvae and CPB larvae in artificial diet bioassays

	LC_{50} ng CryIII/mm^2 (95% C.I.)	
Strain	SCRW	CPB
EG7231	359 (238-593)	0.23 (0.05-0.49)
EG4961	421 (253-1086)	0.32 (0.19-0.50)
EG7211	9.4% dead	12.5% dead
Control	6.25% dead	3.125 % dead

The following Example 13 relates to studies in which the insecticidal activity of CryIII proteins against coleopteran insects is demonstrably enhanced by the combination of a CryI protein with a CryIII protein. CryIA(c) protein crystals are not toxic to coleopteran insects, but are known to be active against numerous species of lepidopteran insects.

Example 13

Synergistic Enhancement of Insecticidal Activity of CryIII Protein by Adding CryI Protein

A recombinant B.t. strain, EG1269, producing only CryIA(c) protein crystals, was grown on liquid sporulation media using the techniques described above generally in Examples 5-7. Recombinant B.t. strain EG1269 was constructed by introducing plasmid pEG157 into B.t. strain HD73-26. Plasmid pEG157 was made by subcloning the cryIA(c) gene from pEG87 (B.t. strain HD263-6), into the shuttle vector pEG147. The CryIA(c) protein crystals were purified by Renografin gradient and quantified using the SDS-PAGE method mentioned previously. An equal amount of these CryI crystals was added to CryIIIC crystals and the crystal protein mixture was bioassayed on artificial diet against SCRW larvae. The CryIIIC-CryI protein mixture was significantly more toxic than the CryIIIC crystals alone, as is clearly indicated by the data in Table 6.

Table 6

Insecticidal activity of a mixture of CryIIIC and CryIA(c) crystal proteins against SCRW larvae in an artificial diet bioassay

Tr atment	# assays	LC_{50} ng CryIIIC/mm^2 (95% C.I.)
CryIIIC crystals	2	1180 (810-2000)
CryIIIC crystals + CryIA(c) crystals	2	309 (220-500)
CryIA(c) crystals	2	15.6% dead at 571 ng/mm^2
Control mortality		6.25%

To assure the availability of materials to those interested members of the public upon issuance of a patent on the present application deposits of the following microorganisms were made prior to the filing of present application with the ARS Patent Collection, Agricultural Research Culture Collection, Northern Regional Research Laboratory (NRRL), 1815 North University Street, Peoria, Illinois 61064, as indicated in the following Table 7:

Table 7

Bacterial Strain	NRRL Accession No.	Date of Deposit
B.thuringiensis EG2158	B-18213	April 29, 1987
B.thuringiensis HD73-26	B-18508	June 12, 1989
B.thuringiensis EG4961	B-18533	September 13, 1989
B.thuringiensis EG2838	B-18603	February 8, 1990
B.thuringiensis EG7231	B-18627	February 28, 1990
E. coli EG7218	B-18534	September 13, 1989

These microorganism deposits were made under the provisions of the "Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure". All restrictions on the availability to the public of these deposited microorganisms will be irrevocably removed upon issuance of a United States patent based on this application.

The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification as indicating the scope of the invention.

ANNEX M3

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 4, line 27 of the description [1]

A. IDENTIFICATION OF DEPOSIT [3]

Further deposits are identified on an additional sheet [X] [2]

Name of depositary institution [4]

American Research Culture Collection (NRRL)

Address of depositary institution (including postal code and country) [4]

1815 N. University Street
Peoria, Illinois 61604 United States of America

Date of deposit [5]	Accession Number [6]
See Attachment	See Attachment

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet [ ]

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. [X] This sheet was received with the international application when filed (to be checked by the receiving Office)

Nathaniel T. Hayden
PCT INTERNATIONAL SERVICES DIVISION
(Authorized Officer)

[ ] The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

(January 1985)

ATTACHMENT TO FORM PCT/RO/134

CONTINUATION OF "MICROORGANISM" BOX:

page 8, line 8
page 9, line 28
page 12, lines 6 and 8
page 42, line 5

CONTINUATION OF IDENTIFICATION OF DEPOSIT BOX A:

The following microorganisms were deposited in the depository institution listed in Box A on the dates listed below:

Bacterial Strain	NRRL Accession No.	Date of Deposit
B.thuringiensis EG2158	B-18213	April 29, 1987
B.thuringiensis HD73-26	B-18508	June 12, 1989
B.thuringiensis EG4961	B-18533	September 13, 1989
B.thuringiensis EG2838	B-18603	February 8, 1990
B.thuringiensis EG7231	B-18627	February 28, 1990
E. coli EG7218	B-18534	September 13, 1989
** B.thuringiensis EG7231	B-18627N	April 17, 1990

** Please note that the NRRL's sample of original deposit of B-18627 was found not to be equivalent to what was originally deposited by Ecogen Inc. As a result, a new deposit of the same microorganism, designated by Ecogen Inc. as EG7231, was sent to NRRL as a new deposit and given the accession number B-18627N.

CLAIMS

1. A purified and isolated cryIIIC gene having a nucleotide base sequence coding for the amino acid sequence illustrated in Figure 1.

2. A purified and isolated cryIIIC gene according to claim 1 wherein the gene has a coding region extending from nucleotide bases 14 to 1972 in the nucleotide base sequence illustrated in Figure 1.

3. A recombinant plasmid containing the gene of claim 1 or 2.

4. A coleopteran-toxic protein produced by the gene of claim 1 or 2.

5. A biologically pure culture of a bacterium transformed with the recombinant plasmid of claim 3.

6. The bacterium of claim 5 wherein the bacterium is Bacillus thuringiensis.

7. The Bacillus thuringiensis bacterium of claim 6 deposited with the NRRL having accession number NRRL B-18627.

8. An insecticide composition comprising the protein of claim 4 and an agriculturally acceptable carrier.

9. An insecticide composition comprising the bacterium of claim 5, a coleopteran-toxic protein produced by such bacterium, and an agriculturally acceptable carrier.

10. A plant transformed with the gene of claims 1 or 2.

11. The cryIIIC gene of claim 2 wherein the gene or a portion thereof is labeled for use as a hybridization probe.

12. A biologically pure culture of a Bacillus thuringiensis bacterium deposited with the NRRL having accession number NRRL B-18533.

13. A coleopteran-toxic protein characteristic of that made by the Bacillus thuringiensis bacterium of claim 12 and having the amino acid sequence illustrated in Figure 1.

14. An insecticide composition comprising the coleopteran-toxic protein of claim 13, in combination with an agriculturally acceptable carrier.

15. The insecticide composition of claim 14 wherein the coleopteran-toxic protein is contained in a Bacillus thuringiensis bacterium.

16. A method of controlling coleopteran insects comprising applying to a host plant for such insects an insecticidally effective amount of the coleopteran-toxic protein of claim 4.

17. The method of claim 16 wherein the coleopteran-toxic protein is contained in a Bacillus thuringiensis bacterium.

18. The method according to claim 16 wherein the insects are of the genus Diabrotica.

19. A method of controlling coleopteran insects which comprises applying to a host plant for such insects an insecticidally effective amount of the coleopteran-toxic protein of claim 13.

20. The method of claim 19 wherein the coleopteran-toxic protein is contained in a Bacillus thuringiensis bacterium.

21. The method of claim 19 wherein the insects are of the genus Diabrotica.

22. A method of enhancing the insecticidal activity of an insecticidal composition containing a coleopteran-toxic protein comprising incorporating into an insecticidal composition containing CryIII protein an amount of CryI protein effective to enhance the insecticidal activity of the composition against coleopteran insects.

23. The method according to claim 22 wherein the CryIII protein is CryIIIC protein.

24. The method according to claim 23 wherein the CryI protein is CryIA protein.

25. The method according to claim 23 wherein the CryI protein is CryIA(c) protein.

26. The method according to claim 22 wherein the CryIII protein and the CryI protein are present in approximately equal amounts.

27. The method according to any of claims 22 through 26 wherein the composition has enhanced insecticidal activity against insects of the genus Diabrotica.

28. An insecticide composition useful against coleopteran insects comprising the coleopteran-toxic protein of claim 13 and a CryI protein, the CryI protein being present in an amount effective to enhance the insecticidal activity of the composition against coleopteran insects.

29. The composition of claim 28 wherein the CryI protein is CryIA protein.

30. The composition of claim 28 wherein the CryI protein is CryIA(c) protein.

31. The composition of claim 28 wherein the coleopteran-toxic protein and CryI protein are present in approximately equal amounts.

FIGURE 1A

```
        10         20         30         40         50         60
GGGAGGAAGAAAAATGAATCCAAACAATCGAAGTGAACATGATACGATAAAGGTTACACC
  RBS          MetAsnProAsnAsnArgSerGluHisAspThrIleLysValThrPr

        70         80         90        100        110        120
TAACAGTGAATTGCAAACTAACCATAATCAATATCCTTTAGCTGACAATCCAAATTCAAC
oAsnSerGluLeuGlnThrAsnHisAsnGlnTyrProLeuAlaAspAsnProAsnSerTh

       130        140        150        160        170        180
ACTAGAAGAATTAAATTATAAAGAATTTTTAAGAATGACTGAAGACAGTTCTACGGAAGT
rLeuGluGluLeuAsnTyrLysGluPheLeuArgMetThrGluAspSerSerThrGluVa

       190        200        210        220        230        240
GCTAGACAACTCTACAGTAAAAGATGCAGTTGGGACAGGAATTTCTGTTGTAGGGCAGAT
lLeuAspAsnSerThrValLysAspAlaValGlyThrGlyIleSerValValGlyGlnIl

       250        260        270        280        290        300
TTTAGGTGTTGTAGGAGTTCCATTTGCTGGGGCACTCACTTCATTTTATCAATCATTTCT
eLeuGlyValValGlyValProPheAlaGlyAlaLeuThrSerPheTyrGlnSerPheLe

       310        320        330        340        350        360
TAACACTATATGGCCAAGTGATGCTGACCCATGGAAGGCTTTTATGGCACAAGTTGAAGT
uAsnThrIleTrpProSerAspAlaAspProTrpLysAlaPheMetAlaGlnValGluVa

       370        380        390        400        410        420
ACTGATAGATAAGAAAATAGAGGAGTATGCTAAAAGTAAAGCTCTTGCAGAGTTACAGGG
lLeuIleAspLysLysIleGluGluTyrAlaLysSerLysAlaLeuAlaGluLeuGlnGl

       430        440        450        460        470        480
TCTTCAAAATAATTTCGAAGATTATGTTAATGCGTTAAATTCCTGGAAGAAAACACCTTT
yLeuGlnAsnAsnPheGluAspTyrValAsnAlaLeuAsnSerTrpLysLysThrProLe

       490        500        510        520        530        540
AAGTTTGCGAAGTAAAAGAAGCCAAGATCGAATAAGGGAACTTTTTTCTCAAGCAGAAAG
uSerLeuArgSerLysArgSerGlnAspArgIleArgGluLeuPheSerGlnAlaGluSe

       550        560        570        580        590        600
TCATTTTCGTAATTCCATGCCGTCATTTGCAGTTTCCAAATTCGAAGTGCTGTTTCTACC
rHisPheArgAsnSerMetProSerPheAlaValSerLysPheGluValLeuPheLeuPr

       610        620        630        640        650        660
AACATATGCACAAGCTGCAAATACACATTTATTGCTATTAAAAGATGCTCAAGTTTTTGG
oThrTyrAlaGlnAlaAlaAsnThrHisLeuLeuLeuLeuLysAspAlaGlnValPheGl

       670        680        690        700        710        720
AGAAGAATGGGGATATTCTTCAGAAGATGTTGCTGAATTTTATCATAGACAATTAAAACT
yGluGluTrpGlyTyrSerSerGluAspValAlaGluPheTyrHisArgGlnLeuLysLe

       730        740        750        760        770        780
TACACAACAATACACTGACCATTGTGTTAATTGGTATAATGTTGGATTAAATGGTTTAAG
uThrGlnGlnTyrThrAspHisCysValAsnTrpTyrAsnValGlyLeuAsnGlyLeuAr

       790        800        810        820        830        840
AGGTTCAACTTATGATGCATGGGTCAAATTTAACCGTTTTCGCAGAGAAATGACTTTAAC
gGlySerThrTyrAspAlaTrpValLysPheAsnArgPheArgArgGluMetThrLeuTh

       850        860        870        880        890        900
TGTATTAGATCTAATTGTACTTTTCCCATTTTATGATATTCGGTTATACTCAAAAGGGGT
rValLeuAspLeuIleValLeuPheProPheTyrAspIleArgLeuTyrSerLysGlyVa
```

FIGURE 1B

```
      910        920        930        940        950        960
TAAAACAGAACTAACAAGAGACATTTTTACGGATCCAATTTTTTCACTTAATACTCTTCA
lLysThrGluLeuThrArgAspIlePheThrAspProIlePheSerLeuAsnThrLeuGl
                                  BamHI
      970        980        990       1000       1010       1020
GGAGTATGGACCAACTTTTTTGAGTATAGAAAACTCTATTCGAAAACCTCATTTATTTGA
nGluTyrGlyProThrPheLeuSerIleGluAsnSerIleArgLysProHisLeuPheAs

     1030       1040       1050       1060       1070       1080
TTATTTACAGGGGATTGAATTTCATACGCGTCTTCAACCTGGTTACTTTGGGAAAGATTC
pTyrLeuGlnGlyIleGluPheHisThrArgLeuGlnProGlyTyrPheGlyLysAspSe

     1090       1100       1110       1120       1130       1140
TTTCAATTATTGGTCTGGTAATTATGTAGAAACTAGACCTAGTATAGGATCTAGTAAGAC
rPheAsnTyrTrpSerGlyAsnTyrValGluThrArgProSerIleGlySerSerLysTh

     1150       1160       1170       1180       1190       1200
AATTACTTCCCCATTTTATGGAGATAAATCTACTGAACCTGTACAAAAGCTAAGCTTTGA
rIleThrSerProPheTyrGlyAspLysSerThrGluProValGlnLysLeuSerPheAs
                                                    HindIII
     1210       1220       1230       1240       1250       1260
TGGACAAAAAGTTTATCGAACTATAGCTAATACAGACGTAGCGGCTTGGCCGAATGGTAA
pGlyGlnLysValTyrArgThrIleAlaAsnThrAspValAlaAlaTrpProAsnGlyLy

     1270       1280       1290       1300       1310       1320
GGTATATTTAGGTGTTACGAAAGTTGATTTTAGTCAATATGATGATCAAAAAAATGAAAC
sValTyrLeuGlyValThrLysValAspPheSerGlnTyrAspAspGlnLysAsnGluTh

     1330       1340       1350       1360       1370       1380
TAGTACACAAACATATGATTCAAAAAGAAACAATGGCCATGTAAGTGCACAGGATTCTAT
rSerThrGlnThrTyrAspSerLysArgAsnAsnGlyHisValSerAlaGlnAspSerIl

     1390       1400       1410       1420       1430       1440
TGACCAATTACCGCCAGAAACAACAGATGAACCACTTGAAAAAGCATATAGTCATCAGCT
eAspGlnLeuProProGluThrThrAspGluProLeuGluLysAlaTyrSerHisGlnLe

     1450       1460       1470       1480       1490       1500
TAATTACGCGGAATGTTTCTTAATGCAGGACCGTCGTGGAACAATTCCATTTTTTACTTG
uAsnTyrAlaGluCysPheLeuMetGlnAspArgArgGlyThrIleProPhePheThrTr

     1510       1520       1530       1540       1550       1560
GACACATAGAAGTGTAGACTTTTTTAATACAATTGATGCTGAAAAGATTACTCAACTTCC
pThrHisArgSerValAspPhePheAsnThrIleAspAlaGluLysIleThrGlnLeuPr

     1570       1580       1590       1600       1610       1620
AGTAGTGAAAGCATATGCCTTGTCTTCAGGTGCTTCCATTATTGAAGGTCCAGGATTCAC
oValValLysAlaTyrAlaLeuSerSerGlyAlaSerIleIleGluGlyProGlyPheTh

     1630       1640       1650       1660       1670       1680
AGGAGGAAATTTACTATTCCTAAAAGAATCTAGTAATTCAATTGCTAAATTTAAAGTTAC
rGlyGlyAsnLeuLeuPheLeuLysGluSerSerAsnSerIleAlaLysPheLysValTh

     1690       1700       1710       1720       1730       1740
ATTAAATTCAGCAGCCTTGTTACAACGATATCGTGTAAGAATACGCTATGCTTCTACCAC
rLeuAsnS rAlaAlaLeuLeuGlnArgTyrArgValArgIleArgTyrAlaSerThrTh

     1750       1760       1770       1780       1790       1800
TAACTTACGACTTTTTGTGCAAAATTCAAACAATGATTTTCTTGTCATCTACATTAATAA
rAsnLeuArgLeuPheValGlnAsnSerAsnAsnAspPheLeuValIleTyrIleAsnLy
```

FIGURE 1c

```
      1810       1820       1830       1840       1850       1860
ACTATGAATAAAGATGATGATTTAACATATCAAACATTTGATCTCGCAACTACTAATTC
sThrMetAsnLysAspAspAspLeuThrTyrGlnThrPheAspLeuAlaThrThrAsnSe

      1870       1880       1890       1900       1910       1920
TAATATGGGGTTCTCGGGTGATAAGAATGAACTTATAATAGGAGCAGAATCTTTCGTTTC
rAsnMetGlyPheSerGlyAspLysAsnGluLeuIleIleGlyAlaGluSerPheValSe

      1930       1940       1950       1960       1970
TAATGAAAAAATCTATATAGATAAGATAGAATTTATCCCAGTACAATTGTAA
rAsnGluLysIleTyrIleAspLysIleGluPheIleProValGlnLeuEnd
```

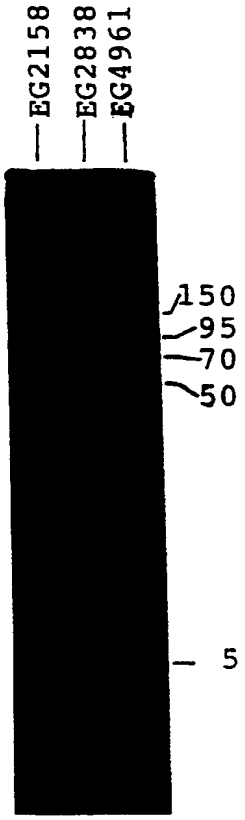


FIG. 2

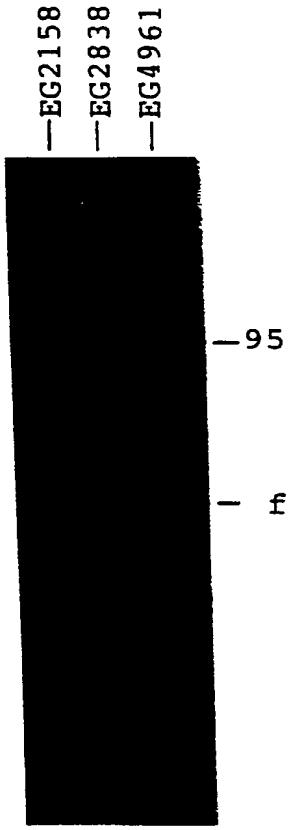


FIG. 3

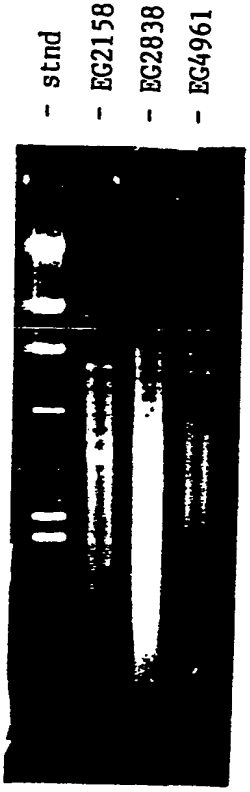


FIG. 4

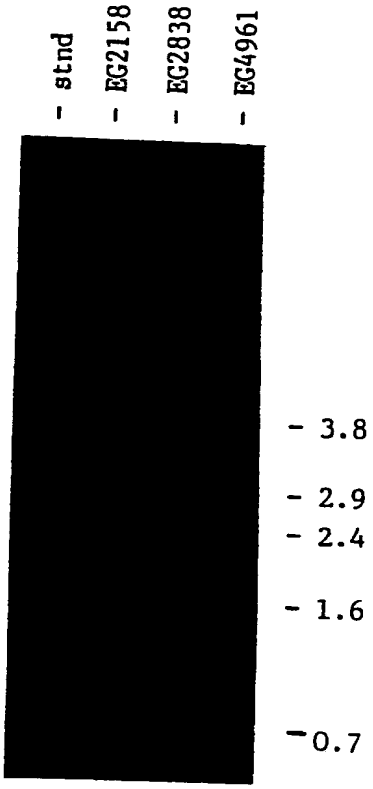


FIG. 5

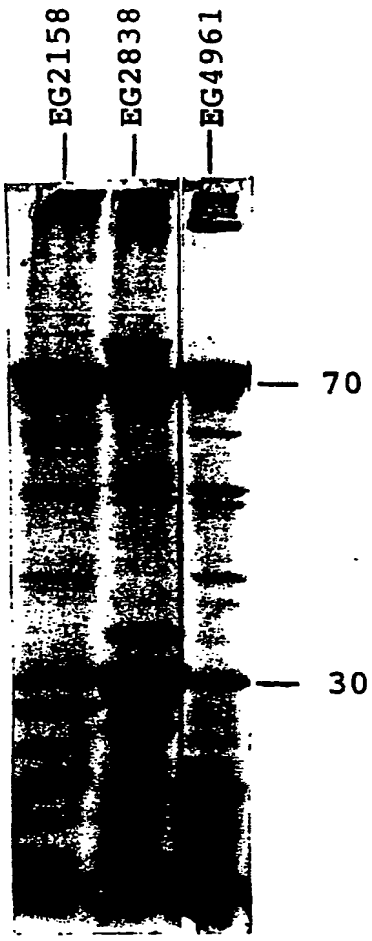
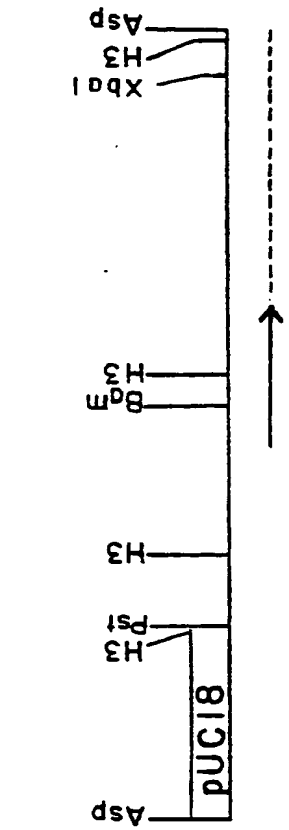
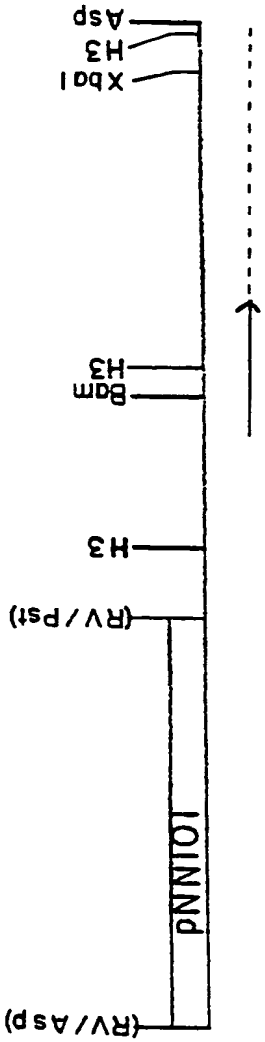


FIG. 6



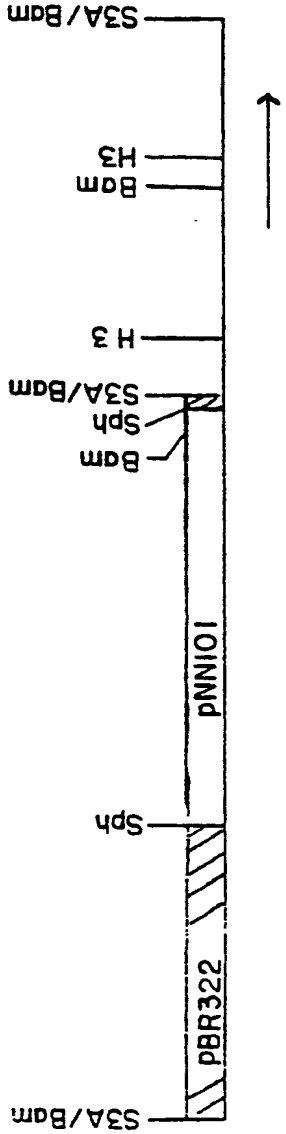
pEG258

FIG. 7



pEG260

FIG. 8



pEG269

FIG. 9

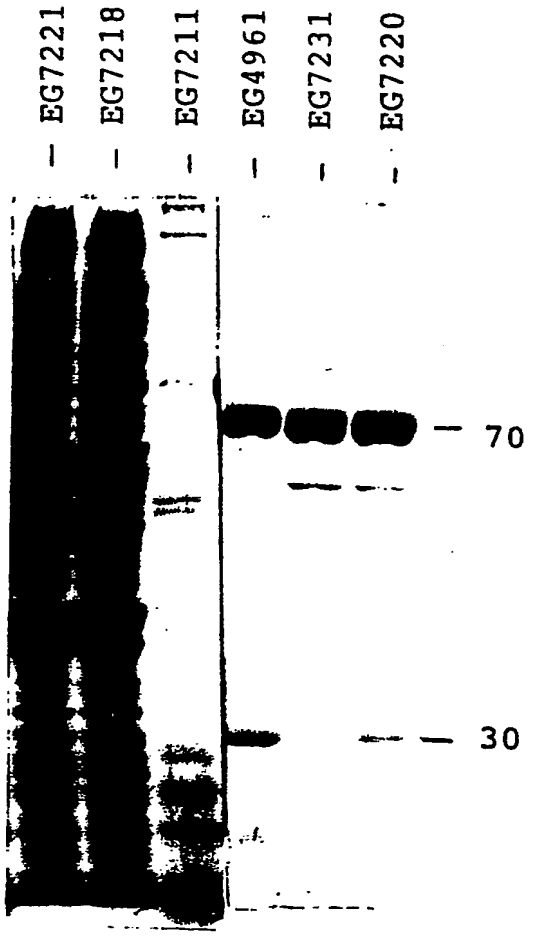


FIG. 10